LYSOSOMAL DESTABILIZATION IN RETINAL PIGMENT EPITHELIAL CELLS ACTIVATES THE NLRP3 INFLAMMASOME AND INDUCES IL-1ß SECRETION

LYSOSOMAL DESTABILIZATION IN RETINAL PIGMENT EPITHELIAL CELLS ACTIVATES THE NLRP3 INFLAMMASOME AND INDUCES IL-1β SECRETION

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Wen Allen Tseng

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Dissertation Advisor: Dr. Patricia Ann D'Amore Wen Allen Tseng

Lysosomal destabilization in retinal pigment epithelial cells activates the NLRP3 inflammasome and induces IL-1β secretion

ABSTRACT

Age-related macular degeneration (AMD) is a leading cause of visual impairment and blindness, affecting over 30 million people worldwide. It is characterized by the appearance of insoluble deposits known as drusen in the outer retina, between the retinal pigment epithelium (RPE) and Bruch's membrane. Drusen are heterogeneously composed of many compounds, including cholesterol, amyloid-β, and complement proteins. AMD also involves the accumulation of pigments collectively termed lipofuscin in RPE lysosomes. The underlying causes of AMD are unknown, but studies have implicated inflammatory processes in its pathogenesis.

The NLRP3 inflammasome is a multiprotein complex, consisting of NLRP3, ASC, and caspase-1, that plays an important role in inflammation. Inflammasome assembly activates caspase-1 via proteolysis of its precursor, procaspase-1. Caspase-1 mediates the maturation of inflammatory cytokines such as IL-1β, as well as a form of cell death called pyroptosis. A myriad of chemically diverse agonists are known to activate the NLRP3 inflammasome, including insoluble particles and crystals, which destabilize phagolysosomes following internalization. As lysosomes are disrupted by the phagocytosis of certain drusen components, such as amyloid-β, or the detergent-like effects of the lipofuscin constituent A2E, I sought to evaluate the hypothesis that the destabilization of RPE lysosomes activates the NLRP3 inflammasome.

Immunohistochemistry of human outer retinal sections revealed that NLRP3 was expressed in the RPE of AMD-affected eyes, but was not detectable in age-matched control retinas. The NLRP3 inflammasome components NLRP3, ASC, and procaspase-1 were expressed in the human RPE cell line ARPE-19 and in primary human RPE cells. Expression of

the IL-1β precursor, pro-IL-1β, in RPE cells was induced by treatment with NF-κB agonists such as IL-1α. Disruption of RPE lysosomes using the lysosomotropic agent L-leucyl-L-leucine methyl ester induced inflammasome activation, as evidenced by caspase-1 activation, processing and release of IL-1β, and pyroptotic cell death. These results suggest a mechanism of AMD pathogenesis in which molecular changes associated with AMD lead to lysosomal damage, activating the NLRP3 inflammasome in RPE cells and mediating vision loss by inducing IL-1β production and cell death. Therefore, the NLRP3 inflammasome may be a key mediator of AMD pathology and a potential target for therapeutic intervention.

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LIST OF ABBREVIATIONS

A2E	N-retinylidene-N-retinylethanolamine
Aβ	amyloid-β
ABC-AP	avidin-biotin-alkaline phosphatase complex
AGE	advanced glycation end-product
AIM2	absent in melanoma 2
AMD	age-related macular degeneration
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
BLamD	basal laminar deposits
BLinD	basal linear deposits
BrM	Bruch's membrane
BSA	bovine serum albumin
CAPS	cryopyrin-associated periodic syndromes
CARD	caspase recruitment domain
CC	choriocapillaris
CEP	carboxyethylpyrrole
CFH	complement factor H
CNV	choroidal neovascularization
DAMP	damage-associated molecular pattern
DPP-I	dipeptidyl peptidase I
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
DSS	dextran sodium sulfate
FBS	fetal bovine serum

FCAS	familial cold autoinflammatory syndrome
FLICA	fluorescent labeled inhibitor of caspases
GA	geographic atrophy
hfRPE	human fetal RPE
HSA	human serum albumin
IL	interleukin
IL-1R	IL-1 receptor
LDH	lactate dehydrogenase
Leu-Leu-OMe	L-leucyl-L-leucine methyl ester
LPS	lipopolysaccharide
MDP	muramyl dipeptide
MSA	mouse serum albumin
MSU	monosodium urate
MWS	Muckle-Wells syndrome
NAIP	NLR family, apoptosis inhibitory protein
NLR	nucleotide-binding domain, leucine-rich repeat-containing protein
NOMID	neonatal-onset multisystem inflammatory disease
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PRR	pattern recognition receptor
ROS	reactive oxygen species
RPE	retinal pigment epithelium
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interfering RNA
siNLRP3	siRNAs targeting NLRP3

SNP	single-nucleotide polymorphism
T2DM	type 2 diabetes mellitus
T3SS	type III secretion system
T4SS	type IV secretion system
TBS-T	Tris-buffered saline with 0.1% Tween 20
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
TXNIP	thioredoxin-interacting protein
VEGF	vascular endothelial growth factor

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CHAPTER 1

GENERAL INTRODUCTION

PREFACE

In this chapter, Figure 1 has been adapted with permission from the article, “Age-related macular degeneration and the extracellular matrix,” by L.V. Johnson and D.H. Anderson in *The New England Journal of Medicine*, July 22, 2004, Vol. 351, No. 4, pages 320-322, the copyright of which is held by the Massachusetts Medical Society. Figure 2 has been adapted with permission from the article, “NLRP3 inflammasome activation in retinal pigment epithelial cells by lysosomal destabilization: implications for age-related macular degeneration,” by W.A. Tseng, T. Thein, K. Kinnunen, K. Lashkari, M.S. Gregory, P.A. D’Amore, and B.R. Ksander in *Investigative Ophthalmology & Visual Science*, January 2013, Vol. 54, No. 1, pages 110-120, the copyright of which is held by The Association for Research in Vision and Ophthalmology, Inc.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of vision loss in individuals of 50 years of age and over in industrialized nations and impairs the vision of more than 30 million people globally (Ambati et al., 2013; Cangemi, 2007; Coleman et al., 2008; Hu et al., 2013; Khandhadia et al., 2012; Parmeggiani et al., 2012). In the United States, roughly 15 million people have AMD, with over 1.7 million individuals suffering from an advanced form of the disease (Friedman et al., 2004; Gryziewicz, 2005). As the average age of the American population increases, it is projected that three million people in the United States will have advanced AMD by the year 2020 (Friedman et al., 2004).

AMD is characterized by pathological changes to the cells and structures of the outer retina, resulting in damage to the central region of the retina known as the macula. The macula contains the fovea and foveola, areas rich in cone photoreceptors, and is responsible for the high acuity central vision that is critical for activities such as reading, driving, and facial recognition (Ambati et al., 2013). Deterioration of central vision from AMD, therefore, has a severely adverse impact on quality of life. However, despite intensive research, the underlying etiology of AMD remains unknown (Liu et al., 2013).

AMD can occur in two forms, often referred to as “dry” and “wet.” Dry AMD, also called atrophic or non-exudative AMD, accounts for over 85% of all AMD and is responsible for 10% of all vision loss associated with AMD. Dry AMD develops slowly over many years and can go undetected in its early stages (Bhutto and Lutty, 2012; Buschini et al., 2011). Its hallmark feature is the presence of yellow-to-white deposits known as “drusen” (German for “geode”) between the basal surface of the retinal pigment epithelium (RPE) and the multilayered extracellular matrix known as Bruch’s membrane (BrM) (Figure 1) (de Jong, 2006; Luibl et al., 2006). Dry AMD is also associated with a thinning or atrophy of the RPE (Penfold et al., 2001). The RPE is responsible for supporting the survival and function of photoreceptors, which lie on

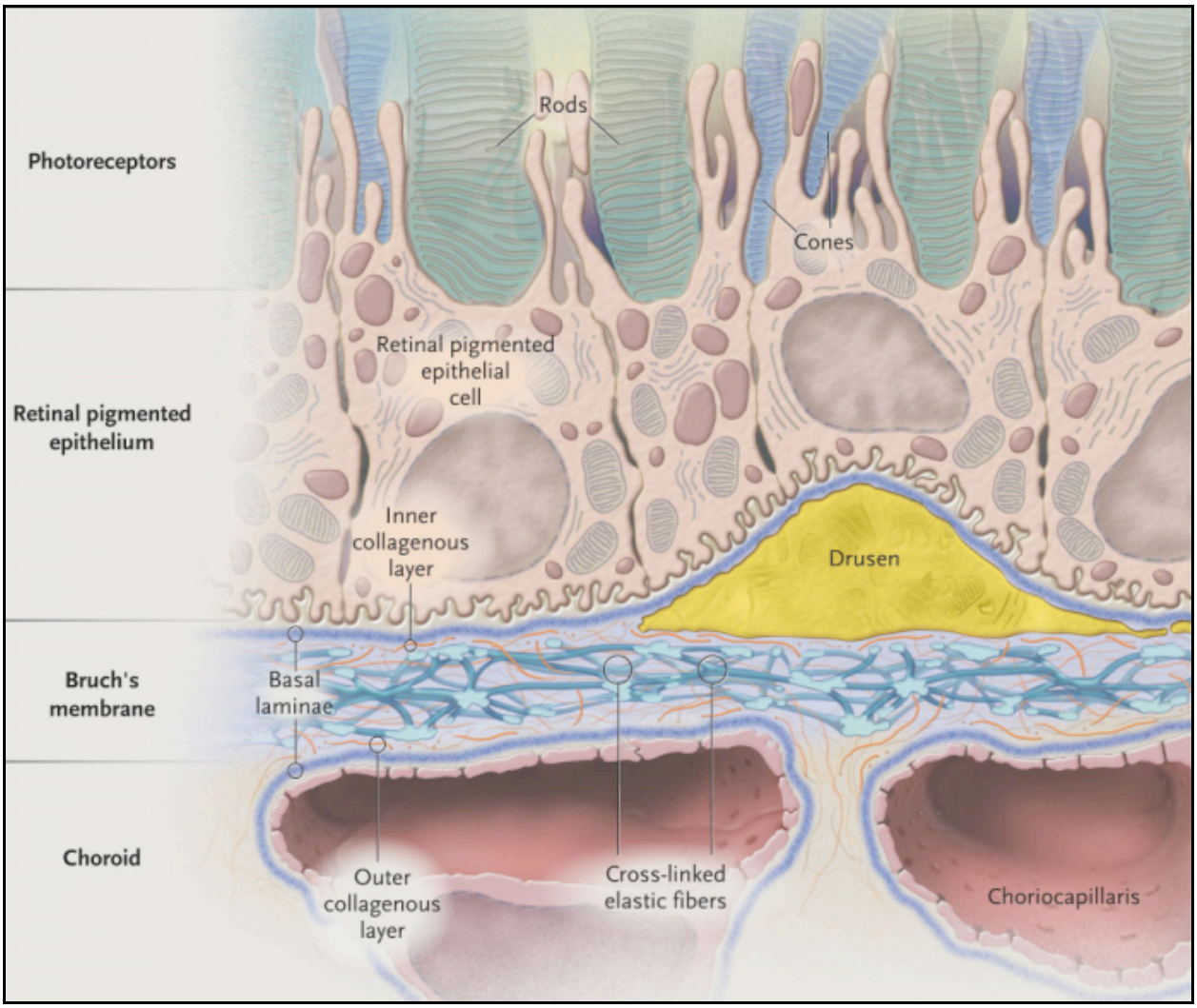

Figure 1. Diagram of the outer retina with drusen accumulation. AMD involves pathological changes in the outer retina, consisting of light-sensing photoreceptors and the RPE. RPE cells maintain the health and function of the receptors, which lie on the apical surface of the RPE. The outer retina is nourished by blood flow from the vascular bed known as the choroid and its associated capillary layer, the choriocapillaris. Between the basal surface of the RPE and the choroid lies BrM, a multilayered extracellular matrix. In aging eyes, deposits referred to as drusen frequently accumulate between the basal lamina of the RPE and the inner collagenous layer of BrM. Drusen are the hallmark feature of AMD and are correlated with AMD progression. In dry AMD, RPE cells degenerate, resulting in the death of the photoreceptors they maintain, causing vision loss. In wet AMD, new blood vessels grow from the choroid, break through BrM, and invade the sub-RPE or subretinal space. Leakage of fluid from these nascent vessels causes vision loss. Reproduced with permission from *New England Journal of Medicine* 351(4): 320-322. Copyright © 2004 Massachusetts Medical Society.

the apical side of the RPE (Bhutto and Lutty, 2012). When dry AMD progresses to its advanced form, called geographic atrophy (GA), patch-like regions of RPE cells deteriorate in the macula, leading to the death of the photoreceptor cells that they maintain (Anderson et al., 2013). As these patches grow and become confluent, they result in central scotomas, areas of vision loss in the center of one's field of view (Ambati and Fowler, 2012; Wang et al., 2009). Despite extensive efforts, there are currently no available treatment options for dry AMD (An et al., 2006). It is likely that the identification of key molecules involved in the pathogenesis of GA will be required for the development of therapeutics effective against this form of AMD.

Wet AMD, also called neovascular or exudative AMD, is an advanced form of AMD that involves choroidal neovascularization (CNV). CNV is characterized by the growth of blood vessels from the choroid, the vascular bed that supplies the outer retina with oxygen and nutrients, and its capillary layer, the choriocapillaris (CC). The nascent vessels penetrate BrM and invade the sub-RPE or subretinal space (Campochiaro, 2013), and because they are highly permeable, these vessels leak fluid that can distort vision or cause detachment of the retina (de Jong, 2006). Wet AMD can progress very rapidly, potentially causing blindness within a matter of months (Munk et al., 2012). Although wet AMD accounts for a minority of total AMD, it is responsible for approximately 90% of severe vision loss caused by AMD (Donoso et al., 2006). Treatments are available for patients with neovascular AMD, primarily in the form of anti-angiogenic agents that target vascular endothelial growth factor (VEGF) and are injected intravitreally (Miller, 2010). These treatments are often able to slow or halt vision loss caused by neovascular AMD, and in some cases can lead to limited recovery of visual acuity (Fong and Lai, 2013). However, not all patients with neovascular AMD respond to anti-VEGF therapy. Nevertheless, even though anti-VEGF agents are far from a cure for neovascular AMD, they can provide substantial improvements in quality of life for many patients.

Over the past several years, an increasing body of evidence has accumulated that implicates a role for inflammation in the pathogenesis of both wet and dry AMD. For example,

many molecules present in drusen participate in inflammation (Hageman et al., 2001). Arguably, the most compelling evidence for an inflammatory contribution to AMD is the fact that AMD susceptibility is strongly associated with single-nucleotide polymorphisms (SNPs) in complement factor H, a major regulator of complement-mediated immune responses and inflammation (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). It is also becoming apparent that an inflammatory state called "para-inflammation," which is milder in intensity than frank inflammation, participates in the development of AMD (Buschini et al., 2011; Medzhitov, 2008; Xu et al., 2009).

Recent studies have implicated a molecular platform known as the NLRP3 inflammasome in AMD pathogenesis. Inflammasomes are multiprotein complexes that mediate the processing of the inflammatory cytokines interleukin (IL)-1β and IL-18 into their mature, biologically active forms. The assembly and activation of inflammasomes is induced by stimuli that are often microbial products or associated with cellular damage, such as extracellular adenosine triphosphate (ATP), monosodium urate (MSU), and hyaluronan (Martinon et al., 2006; Yamasaki et al., 2009). Accordingly, inflammasomes play a key role in innate immunity by mediating host defense against microbes as well as tissue and wound repair (Davis et al., 2011; Dupaul-Chicoine et al., 2010; Franchi et al., 2010; Thomas et al., 2009). However, excessive or uncontrolled NLRP3 inflammasome activity leads to a variety of inflammation-mediated diseases. Evidence indicates that the NLRP3 inflammasome is involved in Alzheimer's disease, atherosclerosis, and type 2 diabetes (Duewell et al., 2010; Halle et al., 2008; Masters et al., 2010; Rajamaki et al., 2010). Several recent studies using animal models have implicated the NLRP3 inflammasome in the development of AMD, but work in this area is early and there are even conflicting reports about whether the inflammasome is protective against AMD or contributes to its development (Doyle et al., 2012; Liu et al., 2013; Marneros, 2013; Tarallo et al., 2012). It is clear that a more complete understanding of inflammasomes, and the NLRP3 inflammasome in particular, is essential for elucidating the etiology and pathogenesis of AMD.

Inflammasomes control caspase-1 activation

Inflammasomes are multiprotein complexes that activate caspase-1, originally known as the IL-1β-converting enzyme (Martinon et al., 2002). Like all caspases, caspase-1 is a cysteine protease that cleaves peptide bonds following aspartate residues and is synthesized as an inactive enzyme precursor or zymogen (Lamkanfi and Dixit, 2012). The best-characterized substrates of caspase-1 are the cytosolic precursors of IL-1β and IL-18, referred to as pro-IL-1β and pro-IL-18, respectively (Brydges et al., 2013). Cleavage of pro-IL-1β or pro-IL-18 by caspase-1 generates the corresponding mature, biologically active cytokine, which is then released through an unconventional and incompletely understood secretory pathway (Lopez-Castejon and Brough, 2011). Because of its role in inflammatory cytokine processing, caspase-1 is categorized as an inflammatory caspase, as opposed to an apoptotic caspase.

The inflammatory caspases are caspase-1, -4, -5, and -12 in humans and caspase-1, -11 and -12 in mice (Khare et al., 2010). Human caspase-4 and -5 are orthologous to murine caspase-11, and are believed to be the products of duplication in the caspase-11 gene (Bian et al., 2011). Although caspase-1 is not known to play a role in apoptosis, it mediates a distinct form of programmed cell death called pyroptosis that is independent of the activity of apoptotic caspases such as caspase-3 and -7 (Lamkanfi and Dixit, 2010). Whereas apoptosis preserves the integrity of the plasma membrane, pyroptosis involves the formation of pores in the plasma membrane that result in cellular swelling, lytic cell death, and release of cellular contents (Fink and Cookson, 2006). Thus, whereas apoptotic blebs can be cleared in an “immunologically silent” manner, pyroptosis is inherently proinflammatory (Lamkanfi and Dixit, 2010; Miao et al., 2010a). Both apoptosis and pyroptosis induce DNA fragmentation, but via different mechanisms (Bergsbaken et al., 2009). By regulating caspase-1 activation, inflammasomes are key

regulators of IL-1β and IL-18 maturation, as well as pyroptosis.

Inflammasome assembly is controlled by scaffolding proteins. Most known inflammasome scaffolding proteins are members of the nucleotide-binding domain, leucine-rich repeat-containing protein (NLR) family. The best-characterized inflammasome scaffolds are NLRP1, NLRP3, and NLRC4, as well as the non-NLR family protein absent in melanoma 2 (AIM2), which belongs to the PYHIN family (Rathinam et al., 2012a). NLRs are a class of pattern recognition receptors (PRRs), which allow the innate immune system to detect pathogens, regardless of prior exposure, and to respond to cellular stress or injury (Kanneganti, 2010). To do this, PRRs bind to common microbial motifs known as pathogen-associated molecular patterns (PAMPs) as well as to certain molecules released from injured or dying cells, such as ATP, called danger signals or damage-associated molecular patterns (DAMPs) (Schroder and Tschopp, 2010). PRRs may reside on the cell surface or in the cytoplasm; Toll-like receptors (TLRs) are membrane-associated PRRs, whereas NLRs reside in the cytosol (Bauernfeind et al., 2011a). Each inflammasome scaffolding protein responds to certain sets of PAMPs and/or DAMPs. Inflammasomes are referred to by the name of their scaffolding protein, and the domain architectures of the different scaffolding proteins influence the compositions of the inflammasomes they assemble.

All inflammasome scaffolding proteins contain at least one effector domain, typically at the N-terminus, that allows them to directly or indirectly recruit procaspase-1, the zymogen precursor of caspase-1. This effector domain is either a pyrin domain or a caspase recruitment domain (CARD) (Davis et al., 2011). These domains undergo homotypic association; pyrin domains bind other pyrin domains, and CARDs bind other CARDs. Procaspase-1 possesses a CARD, so scaffolding proteins that also have a CARD, such as NLRP1 and NLRC4, can directly bind procaspase-1 (Latz et al., 2013). Inflammasome scaffolds that lack a CARD, like NLRP3 and AIM2, have a pyrin domain instead. This domain allows them to interact with an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), which has both a

pyrin domain and a CARD. Thus, scaffolds with a pyrin domain can recruit procaspase-1 indirectly via ASC (Schroder and Tschopp, 2010).

Recruitment of procaspase-1 to the inflammasome results in a high local concentration of the zymogen, which is believed to trigger proximity-induced autoproteolysis, generating active caspase-1 (Broz et al., 2010; Yang et al., 1998). The first step of the proteolytic conversion is the cleavage of procaspase-1 into a p35 fragment, which contains the CARD, and a p10 subunit. Next, the p35 fragment is cleaved to yield the CARD and a p20 subunit (Broz et al., 2010). Active caspase-1 is a tetramer comprised of two p20 subunits and two p10 subunits (Mao et al., 1998). Although NLRP1 and NLRC4 can each recruit procaspase-1 independently of ASC via their CARDs, the presence of ASC in these inflammasomes substantially improves caspase-1 activation and processing of IL-1β and IL-18. Addition of ASC to reconstituted NLRP1 inflammasome complexes increases caspase-1 activity by over two-fold (Faustin et al., 2007), and ASC-deficient macrophages produce roughly 50% lower levels of IL-1β upon induction of the NLRC4 inflammasome, compared to wild-type (Miao et al., 2006). However, the mechanism through which ASC interacts with these inflammasomes remains unclear. Following the convention developed by Ting and colleagues, NLRs are named according to their N-terminal effector domain (Ting et al., 2008). NLRs with an N-terminal pyrin domain are designated “NLRP,” whereas NLRs with an N-terminal CARD are referred to as “NLRC.” Human NLRP1 has both a N-terminal pyrin domain and a C-terminal CARD, but is named based on its N-terminal domain.

Once caspase-1 has been activated, it can cleave the IL-1β and IL-18 precursors to produce mature IL-1β and IL-18. Because pro-IL-1β and pro-IL-18 lack signal peptides that would target them to the ER-Golgi secretory pathway, they reside in the cytosol. After processing, release of the mature cytokines occurs through an unconventional secretory pathway that remains poorly understood (Eder, 2009). Although cell membrane disruption and leakage of cytosolic contents due to pyroptosis has been considered as a possible mechanism,

the cytoprotective agent glycine inhibits inflammasome-mediated cell lysis and leakage but does not affect the release of IL-1β (Edgeworth et al., 2002; Qu et al., 2007; Verhoef et al., 2004), indicating that loss of membrane integrity is not a requirement for IL-1β release. Microvesicle shedding, secretory lysosome exocytosis, exosome exocytosis, and transmembrane ATP-binding cassette transporters have all been proposed as mechanisms for IL-1β secretion (Eder, 2009; Lopez-Castejon and Brough, 2011). Intriguingly, caspase-1 has been found to bind pro-IL-1α and FGF-2, both of which lack consensus signal peptides, and facilitate their unconventional secretion (Keller et al., 2008). Although neither pro-IL-1α nor FGF-2 is a proteolytic substrate of caspase-1, the enzymatic activity of caspase-1 is required for their secretion, as either deletion of caspase-1 or treatment with caspase-1 inhibitors abrogates their release. These results indicate that caspase-1 mediates the unconventional secretion of certain proteins and suggest that IL-1β and IL-18 may rely on caspase-1 not only for their processing, but also their release.

Although caspase-1 is the predominant enzyme that processes IL-1β and IL-18, other enzymes can also cleave pro-IL-1β and pro-IL-18 at positions sufficiently close to the site cleaved by caspase-1 and generate bioactive cytokine. Production of IL-1β by neutrophils in response to *Pseudomonas aeruginosa* infection is independent of caspase-1 and inflammasomes and instead relies on serine proteases (Karmakar et al., 2012). Additionally, IL-1β production in a mouse model of necrotizing crescentic glomerulonephritis is substantially reduced in the absence of the neutrophil serine proteases proteinase 3 and neutrophil elastase (Schreiber et al., 2012). Furthermore, treatment of murine dendritic cells with the proapoptotic chemotherapeutic agents doxorubicin and staurosporine induces caspase-8-mediated IL-1β processing and release independently of caspase-1 (Antonopoulos et al., 2013). Thus, production of mature IL-1β does not necessarily mean that caspase-1 has been activated.

Activation and regulation of inflammasomes

The regulation of inflammasome activity involves a “two-signal” mechanism. One signal induces the expression of pro-IL-1β, and the other signal triggers the assembly of the inflammasome via a scaffolding protein, resulting in the proteolytic activation of caspase-1 (Latz et al., 2013). As described above, each inflammasome scaffold is activated by particular PAMPs and/or DAMPs. NLRP1 is induced by muramyl dipeptide (MDP), a component of bacterial peptidoglycan (Faustin et al., 2007). In addition, certain variants of murine NLRP1b, one of the three NLRP1 paralogues in mice, recognize the *Bacillus anthracis* lethal toxin (Boyden and Dietrich, 2006; Franchi et al., 2012; Terra et al., 2010). NLRC4 is activated by flagellin and certain components of bacterial secretion systems (Zhao et al., 2011). Double-stranded DNA (dsDNA) triggers AIM2 (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009), whereas NLRP3 responds to a diverse array of PAMPs and DAMPs (Bauernfeind et al., 2011a; Duewell et al., 2010). However, in order for an activated inflammasome to produce mature IL-1β, another signal must first induce pro-IL-1β expression, a process referred to as “priming” (Hornung and Latz, 2010). Because NF-κB regulates pro-IL-1β expression via a NF-κB-responsive element in the *IL1B* promoter, priming can be accomplished by NF-κB agonists such as the TLR4 ligand lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), and the cytokine IL-1α (Eisenbarth et al., 2008; Hiscott et al., 1993; Tseng et al., 2013). Unlike pro-IL-1β, pro-IL-18 is constitutively expressed in many cell types and therefore does not require priming (Stutz et al., 2009).

Interestingly, most resting cells also require priming signals to induce transcription of the scaffold proteins NLRP3 and AIM2 (Bauernfeind et al., 2009; von Moltke et al., 2013). Thus, in these cells, the presence of an NLRP3 or AIM2 agonist without priming is not sufficient to induce their respective inflammasomes, whereas for the NLRP1 and NLRC4 inflammasomes, inflammasome activation is independent of priming. Like pro-IL-1β, NLRP3 priming is mediated

via NF-κB (Bauernfeind et al., 2009); however, AIM2 expression is induced by type I interferon signaling rather than NF-κB (Rathinam et al., 2010). The PAMPs and DAMPs that stimulate inflammasome assembly are typically different from those that prime cells; it is rare for one signal to serve both functions. It is hypothesized that this two-signal control mechanism helps to limit inadvertent secretion of IL-1β, which is a highly potent pyrogen, and restricts its release to actual instances of infection or injury (Hornung and Latz, 2010).

The mechanisms through which inflammasome scaffolds are activated by their respective signals are still under investigation. Studies in which the NLRP1 inflammasome was biochemically reconstituted have revealed that its minimal components are NLRP1, a ribonucleoside triphosphate, and caspase-1, and that this reconstituted NLRP1 inflammasome can be activated by MDP (Faustin et al., 2007). Though there is currently no direct evidence of MDP-NLRP1 binding, it has been proposed that NLRP1 binds MDP directly and that this interaction triggers NLRP1 oligomerization and caspase-1 activation (Franchi et al., 2012). Additionally, NLRP1 has been shown to undergo autoproteolytic cleavage (D'Osualdo et al., 2011). Studies also indicate that the intracellular PRR protein NOD2 forms a complex with NLRP1 in response to MDP (Hsu et al., 2008); however, it is not known whether these events play a role in NLRP1 induction. Similar to the mechanism proposed for NLRP1 activation, the inflammasome scaffold AIM2 directly binds its ligand, dsDNA (Hornung et al., 2009). AIM2 is a member of the PYHIN family, rather than the NLR family, and therefore contains a pyrin domain and a HIN200 DNA-binding domain (Fernandes-Alnemri et al., 2009). AIM2 is located in the cytosol, where it detects dsDNA from viruses or intracellular bacteria, and where its exposure to self-DNA is restricted under normal conditions (Rathinam et al., 2012a). When the HIN200 domain engages dsDNA, AIM2 activates inflammasome assembly via its pyrin domain (Rathinam et al., 2010).

Whereas AIM2, and possibly NLRP1, are activated by directly binding their ligands, the NLRC4 scaffold interacts with cofactor proteins that serve as the direct receptors (Kofoed and

Vance, 2011; Zhao et al., 2011). In mouse macrophages, flagellin induces NLRC4, and this is mediated by bacterial type III secretion systems (T3SS) and type IV secretion systems (T4SS) that facilitate entry of flagellin into the cytosol where it can interact with NLRC4 cofactor proteins (Miao et al., 2006; Miao and Warren, 2010). T3SS inner rod proteins also trigger murine NLRC4 independently of flagellin (Miao et al., 2010b). The receptors that bind these ligands belong to the NLR family, apoptosis inhibitory protein (NAIP) group of proteins. Binding of flagellin by NAIP5 or NAIP6 enables the NAIP to complex with NLRC4, inducing inflammasome assembly. Similarly, T3SS inner rod proteins are physically recognized by NAIP2, which activates NLRC4 (Kofoed and Vance, 2011; Zhao et al., 2011). Humans have only one NAIP homologue, which binds T3SS needle proteins, such as CprI of *Chromobacterium violaceum*, and induces NLRC4 (Zhao et al., 2011).

As mentioned above, the NLRP3 inflammasome is induced by physically and chemically diverse entities. Bacteria such as *Listeria monocytogenes* and *Stapholococcus aureus*, viruses including influenza A, and fungi such as *Candida albicans* have been demonstrated to activate NLRP3 (Allen et al., 2009; Craven et al., 2009; Hise et al., 2009; Kim et al., 2010). NLRP3 also responds to microbial products including bacterial pore-forming toxins and the potassium ionophore nigericin (Franchi et al., 2012; Mariathasan et al., 2006). Additionally, NLRP3 detects many types of DAMPs, such as extracellular ATP, hyaluronan, and an assortment of insoluble substances and particulates, including asbestos fibers, silica crystals, MSU crystals, cholesterol crystals, and amyloid-β (Aβ) (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008; Mariathasan et al., 2006; Yamasaki et al., 2009). The diversity of these activators suggests that they activate a common downstream pathway or set of pathways that lead to NLRP3 induction, rather than binding NLRP3 directly.

Several models for NLRP3 activation have been proposed. A number of NLRP3 agonists trigger potassium efflux, leading to low intracellular potassium levels, which is proposed to activate NLRP3. For example, NLRP3 induction by extracellular ATP is mediated by potassium

efflux resulting from activation of the $P2X_7$ receptor, an ATP-gated cation channel (Mariathasan et al., 2006). Microbial pore-forming toxins and nigericin also cause intracellular potassium depletion, and blocking potassium efflux with high extracellular potassium inhibits NLRP3 inflammasome activity (Jin and Flavell, 2010).

Another model hypothesizes that NLRP3 agonists induce the generation of reactive oxygen species (ROS), leading to NLRP3 activation. In accordance with this hypothesis, ROS scavengers inhibit NLRP3 inflammasome induction by asbestos fibers, MSU crystals, and ATP (Dostert et al., 2008). It has also been found that ROS generated by damaged mitochondria activate NLRP3. Consequently, blocking the autophagic degradation of mitochondria causes ROS-producing mitochondria to accumulate, triggering NLRP3 (Zhou et al., 2011). Thioredoxin-interacting protein (TXNIP), which binds the oxidoreductase thioredoxin under basal conditions, has been proposed to dissociate from thioredoxin in response to elevated ROS and subsequently interact with and activate NLRP3 (Zhou et al., 2010). However, it has also been reported that ROS inhibitors do not block NLRP3 induction, but rather prevent its expression (Bauernfeind et al., 2011b).

A third model proposes a mechanism for NLRP3 activation by crystalline or particulate matter (Figure 2) (Hornung et al., 2008). In this model, these substances must be phagocytosed in order to activate NLRP3, as compounds that interfere with phagocytosis by disrupting cytoskeletal dynamics, such as cytochalasin D, block NLRP3 induction. Following phagocytosis, particulate materials destabilize phagolysosomes, and it is proposed that the leakage of certain lysosomal enzymes into the cytosol induces NLRP3. Disruption of lysosomes without particulate matter via osmotic shock or the lysosomotropic agent L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) also triggers NLRP3. The lysosomal enzymes implicated in NLRP3 induction include the cysteine proteases cathepsin B and cathepsin L (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008). Inhibition or deletion of either enzyme partially but significantly suppresses NLRP3 inflammasome activation by lysosomal destabilization. These findings

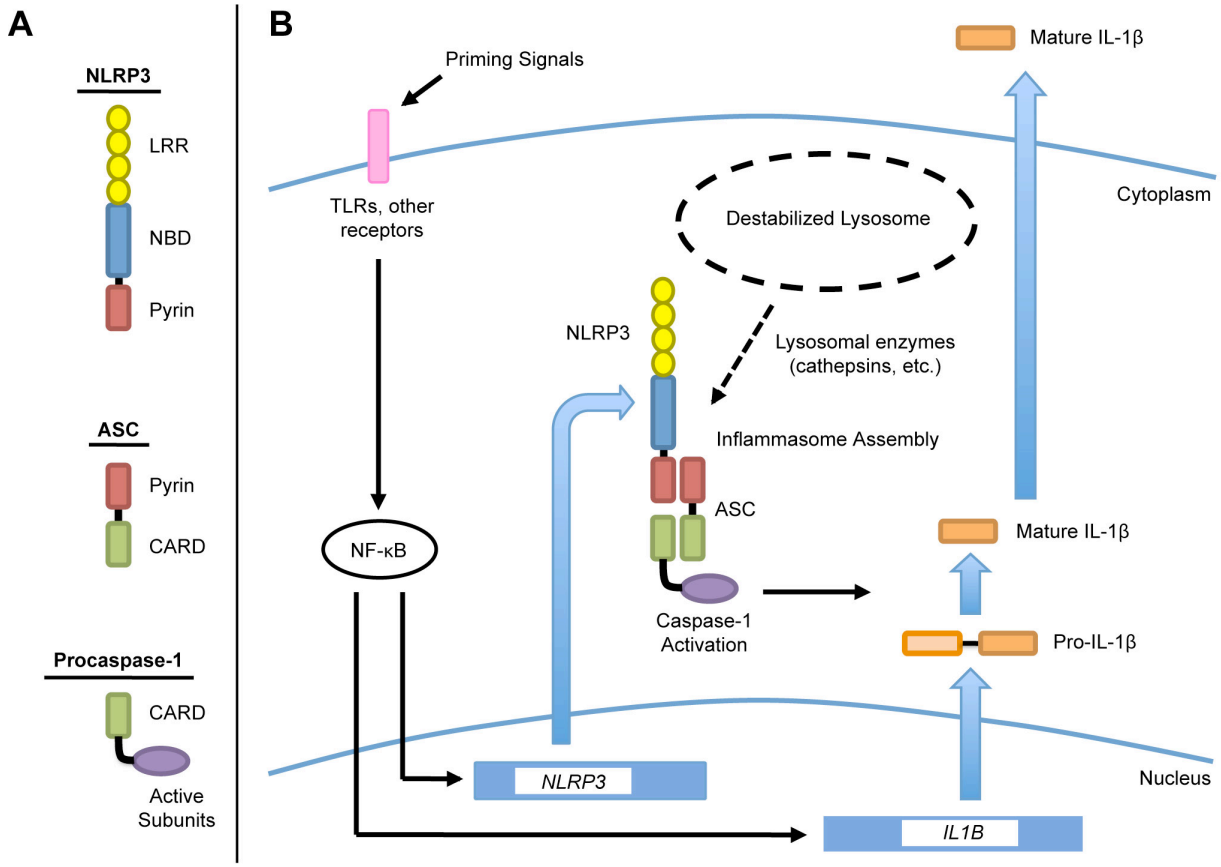

Figure 2. Schematic diagram representing model of NLRP3 inflammasome activation by lysosomal destabilization. (A) Domain architecture of the components of the NLRP3 inflammasome. Inflammasome assembly is mediated by homotypic interactions between pyrin domains on NLRP3 and ASC, and between caspase recruitment domains (CARDs) on ASC and caspase-1. (B) Two-signal model in which priming signals (signal 1) induce expression of NLRP3 and pro-IL-1β via NF-κB. Lysosomal destabilization (signal 2) causes leakage of lysosomal enzymes into the cytosol. These enzymes, such as cathepsins B and L, mediate NLRP3 inflammasome assembly, resulting in caspase-1 activation. Caspase-1 processes pro-IL-1β into mature IL-1β, which is then secreted, and also drives pyroptosis. LRR, leucine-rich repeat; NBD, nucleotide-binding domain.

suggest that cathepsins B and L are redundant with respect to NLRP3 activation, and it is possible that other lysosomal enzymes also perform this function. The mechanism by which lysosomal enzymes activate NLRP3 remains unclear, but a recent study has reported that cathepsins B and L mediate NLRP3 inflammasome assembly by degrading NLRP10, a NLR with inhibitory effects on the NLRP3 inflammasome (Murphy et al., 2013). When lysosomes are intact, NLRP10 binds ASC, preventing it from associating with NLRP3. Following lysosomal damage, cathepsins B and L are released into the cytosol and degrade NLRP10, allowing NLRP3 to interact with ASC and thus inducing inflammasome formation.

It is unclear which model of NLRP3 induction is most accurate, and it is plausible that more than one pathway mediates NLRP3 activation. Furthermore, the processes in these models, potassium efflux, ROS generation, and lysosomal destabilization, are highly interrelated. For example, both lysosomal destabilization and agents that induce potassium efflux, such as extracellular ATP and nigericin, also trigger mobilization of calcium into the cytoplasm from the endoplasmic reticulum (Murakami et al., 2012). Calcium mobilization, in turn, can cause mitochondrial damage and ROS generation. Thus, the interplay among these pathways makes the dissection of their individual contributions challenging.

In addition to the canonical NLRP3 inflammasome consisting of NLRP3, ASC, and caspase-1, evidence indicates that NLRP3 participates in a “non-canonical inflammasome” that is dependent on caspase-11 and is activated in response to “non-canonical” NLRP3 stimuli such as Gram-negative bacteria and cholera toxin B (Kayagaki et al., 2011; Rathinam et al., 2012b). Like the canonical NLRP3 inflammasome, the non-canonical inflammasome mediates IL-1β/IL-18 processing and programmed cell death. The non-canonical inflammasome requires caspase-1 for processing of IL-1β and IL-18, but caspase-1 activation is dependent on caspase-11, in addition to NLRP3 and ASC. Agents that induce the NLRP3 inflammasome independently of caspase-11 are considered canonical and include extracellular ATP, crystalline or particulate matter, pore-forming toxins, and nigericin. Interestingly, NLRP3, ASC, and caspase-1 are

dispensable for the induction of cell death (Kayagaki et al., 2011). Further research is required to obtain a more complete understanding of the mechanisms of non-canonical inflammasome regulation, activation, and function.

The NLRP3 inflammasome in disease

Among the inflammasomes, the NLRP3 inflammasome has the most well characterized role in pathology. Autosomal dominant gain-of-function mutations in the *NLRP3* gene are responsible for a family of autoinflammatory disorders known as cryopyrin-associated periodic syndromes (CAPS), or cryopyrinopathies, referring to NLRP3 by its former name, cryopyrin (Kubota and Koike, 2010; Shinkai et al., 2008). CAPS consist of a spectrum of three disorders of differing severity. The most mild of these is familial cold autoinflammatory syndrome (FCAS), formerly known as familial cold-induced urticaria. Muckle-Wells syndrome (MWS) is of intermediate severity, whereas neonatal-onset multisystem inflammatory disease (NOMID), also known as chronic infantile neurologic cutaneous and articular syndrome, is the most serious. Characteristic symptoms of FCAS include flares of rash, fever, and joint pain induced by exposure to cold. In MWS, these symptoms are more chronic and can occur spontaneously in addition to being provoked by cold or stress. Additionally, up to 60% of MWS patients develop sensorineural hearing loss, and systemic amyloid A amyloidosis occurs in roughly 25% of MWS patients, progressively leading to renal dysfunction (Church et al., 2008; Posch et al., 2012). NOMID, the most severe of the trio, not only causes fever, rash, and joint pain, but also involves inflammation of the central nervous system that, if left untreated, can result in substantial physical disabilities, mental impairment, and neurological issues such as chronic meningitis (Goldbach-Mansky, 2011).

NLRP3 mutations associated with CAPS result in an overactive NLRP3 inflammasome (Shinkai et al., 2008). Consistent with this fact, the symptoms of CAPS are mediated by the IL-1

pathway, and targeted inhibition of IL-1β or the IL-1 receptor (IL-1R) using agents such as the IL-1R antagonist anakinra, the monoclonal anti-IL-1β antibody canakinumab, or the IL-1 "trap" fusion protein rilonacept have shown substantial success in treating CAPS (Kubota and Koike, 2010). Notably, some CAPS patients do not have mutations in *Nlrp3*; approximately 50% of NOMID patients do not exhibit *Nlrp3* mutations (Aksentijevich et al., 2002). It is unclear whether mutations in other inflammasome-related genes are involved in these cases, or if mutations in a separate pathway can also produce CAPS symptoms.

While mutations in NLRP3 can cause illness, wild-type NLRP3 also mediates a number of diseases, many of which involve inflammatory responses to crystalline or particulate matter. For example, gout results from high serum levels of uric acid, leading to the deposition of MSU crystals in joints and surrounding tissues (Martinon et al., 2006). These MSU crystals are internalized by myeloid cells and activate the NLRP3 inflammasome via lysosomal destabilization, resulting in the IL-1β secretion that mediates gout symptoms (Hornung et al., 2008). The related condition pseudogout arises from the deposition of calcium pyrophosphate dihydrate crystals in joints, where they also induce NLRP3-mediated inflammation (Martinon et al., 2006). Similarly, NLRP3 activation by articular hydroxyapatite crystals contributes to the pathogenesis of osteoarthritis (Jin et al., 2011). The lung pathologies silicosis and asbestosis result from the inhalation of crystalline silica dust and asbestos fibers, respectively (Cassel et al., 2008). Lung alveolar macrophages phagocytose these insoluble substances, activating NLRP3. The resulting inflammation induces pulmonary fibrosis, impairing lung function. It is notable that alum crystals, which constitute the most widely used adjuvant in human vaccines, derive their adjuvant properties from their ability to induce the NLRP3 inflammasome (Eisenbarth et al., 2008; Li et al., 2007).

NLRP3 also appears to be involved in age-related diseases associated with chronic inflammation such as Alzheimer's disease. In vitro, NLRP3 is activated in microglia, the myeloid-derived resident immune cells of neural tissues, by phagocytosis of Aβ, a major component of

amyloid plaques associated with Alzheimer's disease (Halle et al., 2008). In vivo, levels of active caspase-1 are significantly higher in brains from Alzheimer's disease patients than those of age-matched control individuals without neurological disease (Heneka et al., 2013). Furthermore, using the APP/PS1 mouse model of Alzheimer's-like pathology, deletion of either *Nlrp3* or *Casp1* protected mice from cognitive impairment and reduced the accumulation of Aβ deposits in mouse brains (Heneka et al., 2013).

Evidence also implicates the NLRP3 inflammasome in the pathogenesis of atherosclerosis (Duewell et al., 2010). In the early lesions of atherosclerosis, cholesterol crystals have been found colocalized with macrophages. Ingestion of cholesterol crystals by macrophages in vitro induces the NLRP3 inflammasome via phagolysosomal destabilization and the activity of cathepsins B and L. Furthermore, the LDLR-deficient mouse model of hypercholesterolemia has been utilized to evaluate the contribution of the NLRP3 inflammasome to atherosclerosis. LDLR-deficient mice were reconstituted with bone marrow from NLRP3-deficient, ASC-deficient, or wild-type mice and fed a high cholesterol diet. Despite similarly high plasma cholesterol levels, mice reconstituted with NLRP3- or ASC-deficient bone marrow are significantly protected from the development of atherosclerotic lesions compared to mice reconstituted with wild-type bone marrow. Contrary to this study, a report utilizing the ApoE-deficient mouse model of hypercholesterolemia found that double deletion of *ApoE* and *Nlrp3*, *Asc*, or *Casp1* does not protect mice from atherogenesis (Menu et al., 2011). However, two other papers have reported that *ApoE*$^{-/-}$*Casp1*$^{-/-}$ double knockout mice have reduced areas of atherosclerotic lesions compared to *ApoE* single knockouts (Gage et al., 2012; Usui et al., 2012). Additional research is warranted to clarify the nature of the discrepancies and to resolve the role of NLRP3 in atherosclerosis.

Growing evidence points to a role for NLRP3 in the pathogenesis of type 2 diabetes mellitus (T2DM). T2DM results from insulin resistance coupled with substantial loss of pancreatic islet β-cell function and mass (Ahren, 2005; Prentki and Nolan, 2006). Several

studies have demonstrated an improvement in glucose tolerance and insulin sensitivity in mice lacking NLRP3 or ASC (Stienstra et al., 2011; Vandanmagsar et al., 2011; Wen et al., 2011; Youm et al., 2011). Evidence suggests that the NLRP3 inflammasome contributes to obesity-induced insulin resistance by mediating IL-1β secretion from adipose tissue macrophages in response to the saturated fatty acid palmitate and/or the fatty acid derivatives known as ceramides (Vandanmagsar et al., 2011; Wen et al., 2011). Furthermore, IL-1β released by pancreatic macrophages or islet cells, which have also been found to express NLRP3 inflammasome components, contributes to β-cell death (Masters et al., 2010; Zhou et al., 2010). High glucose concentrations trigger islet cells to secrete modest levels of IL-1β in an NLRP3-dependent manner (Zhou et al., 2010). This glucose-induced IL-1β production was ROS-mediated, and in accordance with the proposed TXNIP-mediated mechanism of NLRP3 induction by ROS, islet cells from TXNIP-deficient mice secrete substantially less IL-1β than wild-type cells in response to high glucose. Furthermore, T2DM is highly associated with the accumulation of amyloid deposits in pancreatic islets, which contributes to the loss of β-cells. The main constituent of these deposits is islet amyloid polypeptide, which activates the NLRP3 inflammasome in macrophages and dendritic cells, resulting in IL-1β release (Masters et al., 2010). A recent study has also found that omega-3 fatty acids block the induction of the NLRP3 inflammasome in macrophages by a variety of agonists, and that feeding mice the omega-3 fatty acid docosahexaenoic acid inhibits the development of high-fat diet-induced insulin resistance in a NLRP3-dependent manner (Yan et al., 2013).

While the role of the inflammasomes has been most extensively studied in myeloid-derived hematopoietic cells, NLRP3 inflammasome activity in non-myeloid cells has been also been demonstrated. The best-documented role of NLRP3 in non-myeloid cells thus far is in intestinal homeostasis. Inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, are characterized by chronic inflammation within the gastrointestinal tract (Zaki et al., 2011). In studies using the dextran sodium sulfate (DSS) mouse model of colitis, drinking water

is supplemented with DSS, which is toxic to the colonic epithelium (Dupaul-Chicoine et al., 2010). Under normal conditions, the colonic epithelium maintains a barrier that restricts gut microflora to the intestinal lumen. Following exposure to DSS, this protective epithelial barrier is disrupted, enabling intestinal microbes and luminal antigens to enter subepithelial tissues and trigger inflammation (Zaki et al., 2010). Interestingly, deletion of *Nlrp3*, *Asc*, or *Casp1* results in increased susceptibility of mice to DSS-induced colitis, suggesting that the NLRP3 inflammasome plays a protective role (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Hirota et al., 2011; Zaki et al., 2010). Bone marrow transplantation experiments indicate that this effect is due to NLRP3 inflammasome activity in a non-myeloid tissue (Dupaul-Chicoine et al., 2010; Zaki et al., 2010). Levels of IL-18, but not IL-1β, significantly increase following DSS exposure in wild-type, but not *Casp1*-/- mice, and injection of *Casp1*-/- mice with recombinant IL-18 rescues them from DSS-induced colitis, demonstrating that IL-18 is responsible for mediating the protection conferred by the NLRP3 inflammasome. Furthermore, isolated colonic epithelial cells were shown to produce IL-18 via the inflammasome (Zaki et al., 2010). The NLRP3 inflammasome and IL-18 were found to mediate their protective effects by inducing tissue repair via epithelial cell proliferation (Dupaul-Chicoine et al., 2010; Zaki et al., 2010). In addition, NLRP3 inflammasome activity in hematopoietic cells was found to play a protective role against colitis-associated tumorigenesis (Allen et al., 2010). However, other studies have reported that the NLRP3 inflammasome and IL-18 contribute to DSS-induced colitis (Bauer et al., 2010; Siegmund et al., 2001; Sivakumar et al., 2002). These contrasting results are likely due to differences in the genetic backgrounds of the mice used, their intestinal microflora, and the pleiotropic effects of IL-18 (Bauer et al., 2012; Dupaul-Chicoine et al., 2010). Nevertheless, the findings from studies of colitis demonstrate that activation of the NLRP3 inflammasome in non-myeloid cells can contribute significantly to physiological and pathological processes.

Several generalizations can be made regarding the pathologies that involve the NLRP3 inflammasome. First, NLRP3 frequently mediates chronic inflammatory responses to insoluble

deposits. Several of these, such as Alzheimer's disease, atherosclerosis, and T2DM, are age-associated diseases in which insoluble material accumulates over time. Second, although inflammasome activity has primarily been observed in hematopoietic cells of the myeloid lineage, some pathological conditions involve NLRP3 activity in local non-myeloid cells, such as pancreatic islet cells in T2DM and intestinal epithelial cells in colitis; the range of cells capable of NLRP3 inflammasome activity has yet to be elucidated. Finally, although NLRP3 inflammasome activity frequently contributes to pathology, in some instances it does play a protective role.

Features of AMD pathology

The insoluble deposits known as drusen are the hallmarks of AMD, and early AMD is clinically diagnosed based on the detection of drusen and/or pigmentation alterations in the macula by ophthalmoscopy (Seddon et al., 2006). Drusen deposition is localized to the basal side of the RPE, in the vicinity of BrM (Figure 1). BrM, located between the RPE and CC, is an elastic lamina consisting of five layers. In order of their location from the RPE to the CC, these layers are: the RPE basal lamina, the inner collagenous layer, the elastic layer, the outer collagenous layer, and the CC basal lamina. Drusen accumulate between the RPE basal lamina and the inner collagenous layer (Mullins et al., 2000). Drusen can also be classified as "hard" or "soft" based on their appearance. Hard drusen are small with sharp, well-defined edges, whereas soft drusen are larger and have indistinct borders. The number, size, and confluence of drusen are risk factors for AMD progression (Klein et al., 2007). Whereas the presence of one or two small hard drusen in the macula is common in older individuals and confers only minimal likelihood of progression to late AMD, approximately eight or more small hard drusen significantly increase the incidence of soft drusen. In turn, the number and area of soft drusen are associated with a higher risk of developing advanced AMD.

Drusen are heterogeneous in composition, but their most abundant components are lipids. Unesterified and esterified cholesterol, phosphatidylcholine, sphingomyelin, and fatty acids are common constituents of drusen, with esterified cholesterol and phosphatidylcholine comprising at least 40% of drusen volume (Wang et al., 2010). In addition to lipids, drusen include apolipoproteins, advanced glycation end-products (AGEs), complement components and factors, vitronectin, immunoglobulins, acute-phase reactants, prothrombin, crystallins and amyloid proteins such as Aβ (Buschini et al., 2011; Crabb et al., 2002; Hageman et al., 2001; Hageman et al., 1999; Rudolf et al., 2008). Notably, many of these molecules have known roles in immune processes and inflammation. The origin of drusen is unresolved, with plasma and the RPE proposed as the most likely sources (Hageman et al., 2001; Wang et al., 2009). Similarly unclear is the relationship between drusen and AMD and whether it is cause or effect. Thus, the role of drusen in the pathogenesis of AMD remains a matter of debate. However, the fact that drusen are insoluble deposits, similar to those found in atherosclerotic plaques or Alzheimer's disease, led to the hypothesis that their internalization could activate the NLRP3 inflammasome via the lysosomal pathway.

Besides drusen, the outer retina of the aging eye also accumulates other deposits, including basal laminar deposits (BLamD), basal linear deposits (BLinD), and reticular pseudodrusen. BLamD are found between the RPE plasma membrane and its basal lamina, and are comprised of basal lamina proteins and long-spacing collagen (Sarks et al., 2007). BLinD, on the other hand, are lipid-rich deposits of membranous material. Similar to drusen, BLinD are located between the RPE basal lamina and the inner collagenous layer of BrM and are associated specifically with AMD (Bhutto and Lutty, 2012; Curcio and Millican, 1999). While drusen, BLinD, and BLamD are located basal to the RPE, reticular pseudodrusen are situated in the subretinal space, between the retina and the apical surface of the RPE (Querques et al., 2013). Also known as subretinal drusenoid deposits, reticular pseudodrusen are associated with late AMD, but not as strongly as soft drusen (Zweifel et al., 2010). As hypothesized for drusen,

the material present in BLamD, BLinD, and reticular pseudodrusen could potentially activate the inflammasome if phagocytosed.

In addition to extracellular deposits, AMD is also associated with the accumulation of lipofuscin in RPE lysosomes. Lipofuscin consists of pigmented granular substances that are not degradable by lysosomal digestion (Terman and Brunk, 2004). Accordingly, lipofuscin is found in the lysosomes of many long-lived, postmitotic cell types including neurons and cardiac myocytes. During their lifetime, RPE cells continuously phagocytose outer segment discs that are shed by photoreceptors as part of the visual cycle (Holz et al., 1999; Sparrow et al., 2010). As such, lipofuscin components, including a variety of bisretinoid compounds, are derived from the outer segment discs. The bisretinoid fluorophore N-retinylidene-N-retinylethanolamine (A2E), which has historically been considered a major constituent of RPE lipofuscin (Sparrow et al., 1999; Wielgus and Roberts, 2012), although this has recently been challenged (Ablonczy et al., 2013), possesses detergent-like properties and has been reported to destabilize lysosomal membranes (Schutt et al., 2002; Sparrow et al., 2006).

Therefore, the development of AMD is associated with exposure of RPE cells to extracellular deposits and intralysosomal lipofuscin, both of which have the potential to cause lysosomal disruption. Moreover, A2E can alkalinize RPE lysosomes, whose acidic pH enables optimal activity of lysosomal degradative enzymes (Bergmann et al., 2004; Holz et al., 1999). The increased pH leads to reduced function of lysosomal enzymes, causing the accumulation of undegraded material. Thus, A2E present in RPE lipofuscin may induce pH-mediated lysosomal dysfunction with subsequent lysosomal membrane destabilization.

Inflammation and AMD

Several lines of evidence suggest a role for inflammation in the pathogenesis of AMD. Although features such as the blood-ocular barrier and immunosuppression by molecules such

as transforming growth factor-β maintain a level of immune privilege in the eye, cells of the innate immune system are nonetheless present in the retina and able to mediate inflammation (Stein-Streilein, 2013). Microglia are the primary resident immune cell of the retina (Lee et al., 2008), and macrophages and dendritic cells can infiltrate the retina from the choroid in situations where the blood-RPE barrier is disrupted (Eter et al., 2008).

Arguably the most compelling evidence for the contribution of inflammation to AMD comes from studies that have strongly implicated the complement system. A major component of the innate immune system, the complement system combats microbial infection by several effector mechanisms including the induction of inflammatory responses. Complement consists of a number of proteins circulating in the blood or bound to cell membranes. Activation of complement occurs via proteolytic cascades and can be initiated via any of three pathways: the classical pathway, the alternative pathway, and the lectin pathway. A number of genetic studies have determined that a SNP in complement factor H (CFH) that changes amino acid residue 402 from a tyrosine to a histidine (Y402H) is associated with a significantly increased risk for developing AMD. Possession of at least one such allele increases the risk for AMD by 2.7-fold and accounts for 40-50% of the population attributable risk for AMD (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). CFH, a key regulator of complement activity, inhibits the proteolytic induction of the alternative pathway in order to protect host cells from complement effector functions such as inflammation. The Y402H variant of CFH binds several of its ligands with reduced affinity, impairing its recruitment and decreasing its effectiveness at restricting complement activation. CFH is known to bind C-reactive protein, which is found in drusen and is elevated in the BrM and CC of AMD patients compared to similarly aged control individuals, and this interaction is weakened by the Y402H polymorphism (Laine et al., 2007; Ormsby et al., 2008; Yu et al., 2007). The Y402H variant also has reduced affinity for glycosaminoglycans, CFH ligands present in BrM and on the RPE surface (Clark et al., 2010). Furthermore, levels of CFH are decreased in the BrM and CC of

AMD patients compared to aged control donors (Bhutto et al., 2011). These findings have led to the hypothesis that autoinflammation mediated by complement dysregulation may promote AMD (Anderson et al., 2010).

In addition, a SNP encoding a phenylalanine (F) at amino acid 412 instead of leucine (L) in TLR3, a PRR involved in innate immune detection of double-stranded RNA (dsRNA), is significantly associated with protection from GA (Yang et al., 2008). Primary cultured RPE cells heterozygous for the TLR3 L and F variants exhibit a 50% reduction in TLR3-mediated cytotoxicity in response to the dsRNA analog polyinosinic:polycytidiylic acid, compared to cells homozygous for the L variant, supporting the concept that the protection from GA conferred by the TLR3 F variant is due to a reduction in TLR3 function. Interestingly, TLR3 induction by dsRNA molecules of at least 21 nucleotides in length protects mice from laser injury-induced CNV (Kleinman et al., 2008). This observation suggests that inflammation may play independent roles in the pathogenesis of GA and neovascular AMD. However, analyses of subfoveal neovascular membranes from patients with wet AMD have detected an abundance of inflammatory cells such as macrophages (Oh et al., 1999). It has been postulated that inflammatory cytokines secreted by macrophages, including IL-1β, may stimulate RPE cells to produce VEGF, thereby promoting CNV. Accordingly, depletion of macrophages using liposomal clodronate reduces the extent of laser-induced CNV in mice (Espinosa-Heidmann et al., 2003; Sakurai et al., 2003).

The seemingly contradictory roles of inflammation in CNV have been proposed to be due to the differential contributions of classically activated and alternatively activated macrophages (Ambati et al., 2013). Stimulation of macrophages with molecules such as LPS or the Th1-type cytokine interferon-γ results in classical activation, also known as M1 activation (Classen et al., 2009). Conversely, the alternatively activated, or M2, macrophage phenotype is induced by the Th2-type cytokines IL-4 and IL-13 (Lawrence and Natoli, 2011; Tugal et al., 2013). M1 macrophages are pro-inflammatory, whereas M2 macrophages are anti-

inflammatory, promote angiogenesis, and facilitate wound healing and tissue repair (Cao et al., 2011; Lawrence and Natoli, 2011). Therefore, contrasting effects of innate immune processes in CNV may be due to the highly divergent roles of M1 and M2 macrophages.

Many constituents of drusen have documented roles in immune-related or inflammatory processes, and drusen deposits and atherosclerotic plaques share many constituents, indicating that AMD may be mediated by inflammatory processes similar to those at play in atherosclerosis (Mullins et al., 2000). For instance, the NLRP3 agonist Aβ is detected both in drusen and in Alzheimer's disease plaques (Halle et al., 2008; Johnson et al., 2002; Liu et al., 2013). AGEs, a category of chemically modified proteins and lipids also present in drusen, as well as in aged RPE and BrM, accumulate with age and promote inflammation (Glenn et al., 2009; Xu et al., 2009; Yamada et al., 2006). AGEs are associated with a number of other age-related diseases including T2DM, atherosclerosis, osteoarthritis, and Alzheimer's disease, further supporting the involvement of inflammation in these pathologies (Reddy and Beyaz, 2006).

Despite the immune privileged nature of the retina, sera from AMD patients contain significantly higher titers of autoantibodies against retinal antigens than that of age-matched controls, leading some to postulate an autoimmune component in AMD (Buschini et al., 2011; Gurne et al., 1991; Mullins et al., 2000; Patel et al., 2005; Penfold et al., 1990). Notably, eyes from AMD patients have been found to contain significantly more proteins adducted to the oxidative stress product carboxyethylpyrrole (CEP) than eyes from age-matched control individuals, and autoantibodies against CEP are substantially elevated in the plasma of AMD patients compared to age-matched individuals without AMD (Crabb et al., 2002; Gu et al., 2003). Immunization of mice with CEP adducted to mouse serum albumin (MSA) results in RPE degeneration reminiscent of GA that correlates with anti-CEP antibody titer (Hollyfield et al., 2008).

It is important to note that many age-associated pathologies of the developed world,

including atherosclerosis, T2DM, and neurodegenerative diseases, involve a low-grade inflammatory process that is intermediate between classic, frank inflammation and the basal state. This milder form of inflammation has been termed para-inflammation (Medzhitov, 2008). Whereas classic inflammation occurs in response to insults such as infection or tissue injury and is normally resolved, para-inflammation appears to be the body's attempt to rectify more moderate perturbations from homeostasis such as tissue stress or malfunction. If dysfunctional conditions persist, para-inflammation is not resolved and becomes chronic and pathological. In the context of AMD, tissue stress and malfunction could be induced by oxidative stress, drusen deposition, AGE accumulation, and RPE lipofuscin buildup, as well as other insults.

The NLRP3 inflammasome and AMD

Myeloid-derived cells such as microglia and macrophages are potential mediators of NLRP3 inflammasome-induced inflammation in AMD. In addition, it has been found that certain non-immune cells that have barrier functions, such as keratinocytes, also express inflammasome components, enabling them to carry out some functions of myeloid cells (Dupaul-Chicoine et al., 2010; Feldmeyer et al., 2007; Watanabe et al., 2007; Yazdi et al., 2010; Yilmaz et al., 2010; Zaki et al., 2010). Similarly, we have shown that RPE cells, which represent the outer retinal barrier and contribute to retinal immune privilege, express the NLRP3 inflammasome (Tseng et al., 2013). Recent reports have provided important information regarding the potential role of the NLRP3 inflammasome in AMD. One study found that drusen isolated from AMD eyes induces the NLRP3 inflammasome in macrophages and dendritic cells in vitro (Doyle et al., 2012). This study also demonstrates that the complement protein C1Q, a constituent of drusen, activates NLRP3 in myeloid-derived cells via phagolysosomal disruption. Two additional reports demonstrate that RPE cells do indeed exhibit NLRP3 inflammasome activity (Tarallo et al., 2012; Tseng et al., 2013). In one of these reports, the accumulation of

RNA transcripts expressed from the *Alu* retrotransposon in RPE cells was shown to trigger NLRP3 via mitochondrial ROS, leading to inflammasome-mediated IL-18 secretion (Tarallo et al., 2012).

Interestingly, these studies have come to contrasting conclusions regarding the involvement of NLRP3 in AMD. In the CEP-MSA immunization mouse model of GA, NLRP3 and cleaved caspase-1 colocalize with the macrophage marker F4/80, implying that NLRP3 inflammasome activity in GA occurs in macrophages (Doyle et al., 2012). In contrast, GA was hypothesized to be mediated by *Alu*-induced NLRP3 inflammasome activity in RPE cells without the requirement of immune cells (Tarallo et al., 2012). While one report implicates NLRP3 inflammasome activity and IL-18 secretion as mediators of GA (Tarallo et al., 2012), the other suggests that NLRP3 and IL-18 protect against CNV induced by laser injury (Doyle et al., 2012). It is possible that NLRP3-mediated IL-18 plays differential roles in GA and neovascular AMD but clearly further investigation is necessary to elucidate this question.

Whereas IL-18 has been implicated in the pathogenesis of AMD, a role for IL-1β in AMD has yet to be identified. Unlike IL-18, processing of IL-1β by the inflammasome requires a priming signal to induce expression of pro-IL-1β. As such, it is possible that the mouse models of AMD pathology used in the studies described above supplied signals that induced NLRP3 inflammasome assembly, but not priming. Consistent with this hypothesis, *Alu* RNA transcript accumulation activates NLRP3 in RPE cells but does not upregulate pro-IL-1β (Tarallo et al., 2012). Thus, I was interested to determine whether RPE cells could secrete IL-1β when appropriate priming signals are provided. The findings that drusen deposits activate NLRP3 in myeloid cells, that the drusen component C1Q induces NLRP3 via lysosomal destabilization, and that RPE cells express NLRP3 inflammasome components suggest that exposure of RPE cells to AMD-related substances such as drusen or lipofuscin may activate NLRP3. Therefore, the work in this thesis aimed to test the hypothesis that lysosomal destabilization activates the NLRP3 inflammasome in RPE cells, and that RPE cells secrete mature IL-1β if primed with an

NF-κB agonist to induce expression of pro-IL-1β prior to inflammasome activation. This was accomplished by: evaluating the expression of NLRP3 in the RPE in vitro and in vivo; determining if pro-IL-1β expression could be induced in RPE by priming; assessing the effect of priming on the levels of individual components of the NLRP3 inflammasome; evaluating the ability of lysosomal destabilization to activate the inflammasome in RPE cells; and characterizing the effects of inflammasome induction in RPE cells, with particular emphasis on IL-1β processing and the cell death mechanism pyroptosis.

CHAPTER 2

EXPRESSION OF NLRP3 INFLAMMASOME COMPONENTS AND INFLAMMASOME PRIMING IN RPE CELLS

PREFACE

The Results and Discussion sections of this chapter are adapted from portions of the article, "NLRP3 inflammasome activation in retinal pigment epithelial cells by lysosomal destabilization: implications for age-related macular degeneration," by W.A. Tseng, T. Thein, K. Kinnunen, K. Lashkari, M.S. Gregory, P.A. D'Amore, and B.R. Ksander in *Investigative Ophthalmology & Visual Science*, January 2013, Vol. 54, No. 1, pages 110-120, the copyright of which is held by The Association for Research in Vision and Ophthalmology, Inc. Although the Results and Discussion of this chapter have been largely reproduced from parts of the original article, adaptations have been made to include data on hfRPE cells in Figures 5 and 6 and in the text of the Results section, to improve readability of the figures when displayed in the format of this dissertation, to maintain uniformity of abbreviations, and to better blend copied segments into the context of the chapter and the dissertation as a whole.

RATIONALE

Whereas inflammasomes have been most heavily studied in hematopoietic cells of the myeloid lineage, such as monocytes and macrophages, non-myeloid cells such as keratinocytes and intestinal epithelial cells have been demonstrated to express components of the NLRP3 inflammasome and to activate the NLRP3 inflammasome (Feldmeyer et al., 2007; Watanabe et al., 2007; Yilmaz et al., 2010; Zaki et al., 2010). Epithelial cells constitute the barriers that separate the body from the external environment or delimit a bodily compartment with restricted immune cell access. It has therefore been suggested that the inflammasome activity of these non-myeloid cells may complement the innate immune system by rapidly responding to pathogens or tissue damage (Yazdi et al., 2010). The RPE helps to maintain the blood-retinal barrier that contributes to retinal immune privilege, and thus it is reasonable that they express and can activate the NLRP3 inflammasome.

Each of the components of the NLRP3 inflammasome, NLRP3, ASC, and procaspase-1, plays a critical role in the inflammasome (Latz et al., 2013). The scaffold protein NLRP3 detects PAMPs and DAMPs via mechanisms that remain unclear. When activated, NLRP3 binds the adaptor protein ASC via homotypic pyrin domain interactions. In turn, ASC recruits procaspase-1 into the complex via their CARDs, leading to the autocatalytic activation of caspase-1, the primary effector enzyme of the inflammasome. Caspase-1 processes cytokine precursors into their mature forms through proteolytic cleavage. The IL-1β precursor, pro-IL-1β, is one of the best-characterized substrates of caspase-1. However, transcription of pro-IL-1β is not constitutive, but requires pro-inflammatory stimulation in a step known as “priming,” which is mediated by NF-κB. Additionally, in many cell types including human and mouse macrophages, NLRP3 is not expressed under basal conditions and also requires priming (Bauernfeind et al., 2009). In the course of the pathogenesis of AMD, it is thought that numerous inflammation-related molecules, including complement proteins and other molecules present in drusen,

accumulate in the vicinity of the RPE. I speculate that these AMD-associated molecules may prime the RPE, leading to the upregulation of pro-IL-1β and NLRP3.

Thus, I propose that RPE cells express the components of the NLRP3 inflammasome, and that priming with NF-κB agonists will induce RPE cells to express pro-IL-1β. This chapter reports the evaluation of the expression of NLRP3 in human eyes, the expression of NLRP3 inflammasome components in cultured RPE cells, and the ability of NF-κB-activating agents to prime RPE cells to express pro-IL-1β. As priming may be required for NLRP3 expression and could also affect ASC and procaspase-1, the effect of priming on the levels of NLRP3 inflammasome components was also examined.

RESULTS

NLRP3 is localized to the RPE and drusen of the eyes of patients with AMD

I first sought to determine whether NLRP3 is expressed by human RPE in vivo, and whether there are qualitative differences between its presence in patients with AMD, in the form of GA or neovascular AMD, and age-matched individuals without AMD. Outer retinal sections were collected from two donors with GA, two donors with neovascular AMD, and three age-matched donors that were unaffected by AMD. The sections were stained with an antibody against NLRP3 or an isotype control (Figure 3). As a positive control of NLRP3 expression, human conjunctival tissue corresponding to each eye was also stained with the same antibodies. NLRP3 (red, arrowheads) was detected in the RPE of eyes affected by either GA or neovascular AMD; there was no staining in sections from the same donors with the control antibody. Several sections from GA-affected eyes contained drusen within the transitional zone, and there was substantial extracellular NLRP3 staining both in choroidal cells beneath BrM and on the basal side of RPE cells adjacent to areas of drusen (Figure 3, A and B). NLRP3 was also

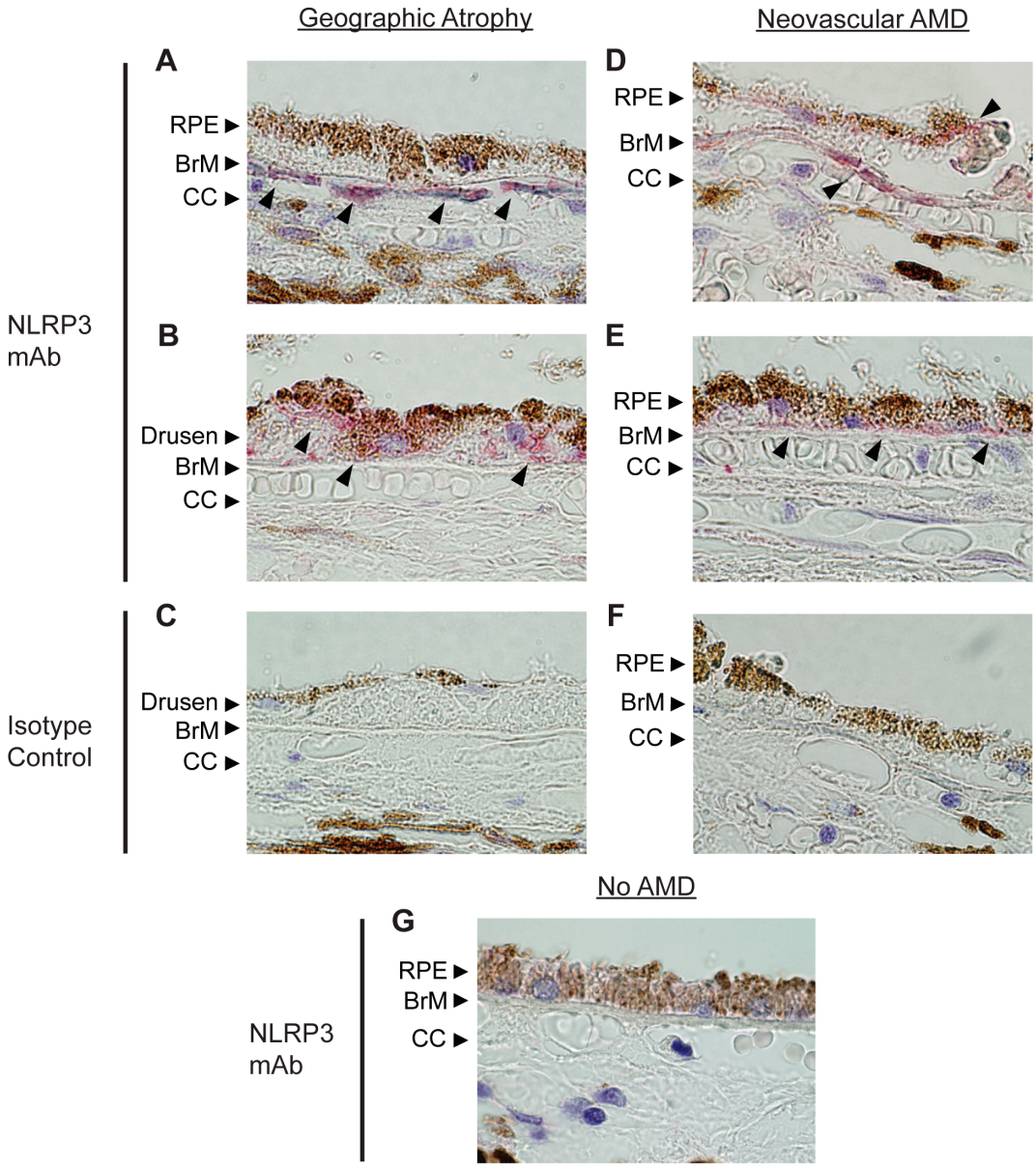

Figure 3. Expression of NLRP3 in AMD-affected eyes. Retinas from donors with (A-C) GA, (D-F) neovascular AMD, or (G) unaffected eyes were stained for NLRP3. NLRP3 (red, arrowheads) was detected in the RPE of eyes affected with either form of AMD. (A, B) Representative sections of the transitional zone in eyes with GA shows areas of staining in the choroidal cells just beneath BrM (A, arrowheads) as well as in the basal side of the RPE adjacent to confluent drusen (B, arrowheads). Some drusen deposits in GA eyes also stained for NLRP3. (D, E) Representative sections just outside the area of choroidal neovascularization display staining in the vicinity of thickened BrM as well as in basal RPE (D, arrowheads). NLRP3 staining was also seen within the thickened BrM representing basal linear deposits (E, arrowheads). (C, F) There was no detectable signal in sections from the same donors stained with an isotype control. (G) A representative section from an age-matched donor unaffected by AMD did not exhibit any NLRP3 expression.

detected extracellularly in the vicinity of thickened BrM (Figure 3D) and associated with basal linear deposits (Figure 3E) in eyes with neovascular AMD outside the area of choroidal neovascularization as well as in basal RPE (Figure 3D). NLRP3 was only detected at sites of GA or neovascular lesions in AMD eyes and was not observed in the RPE of lesion-free areas of the retina. In contrast to AMD-affected eyes, there was not detectable NLRP3 staining in outer retinal sections from eyes of age-matched donors unaffected by AMD.

NLRP3 is expressed in human RPE cells in vitro

Motivated by the observation of NLRP3 in RPE of AMD patients, I investigated the expression of NLRP3 by RPE cells in vitro using the human RPE cell line ARPE-19. ARPE-19 cells were cultured on Transwell membranes for four weeks to allow them to polarize and form tight junctions, characteristics of differentiated RPE in vivo. Immunocytochemical localization revealed NLRP3 distributed in a punctate pattern throughout the cells (Figure 4A). Confocal microscopy indicated that NLRP3 was preferentially localized toward the basal aspect of the cells (Figure 4A).

Next, I evaluated NLRP3 protein expression in ARPE-19 cells by immunoblotting (Figure 4B). Two methods were used to definitively identify the NLRP3 band in western blotting: specific knockdown of NLRP3 with siRNA against NLRP3 and the inclusion of a positive control that consisted of cells that were transfected with NLRP3. ARPE-19 cells cultured in plastic wells were transfected with a pool of 4 siRNAs against NLRP3 (siNLRP3) or a non-targeting control siRNA pool. Table 1 lists the target sequences of the siRNAs in each pool. A band migrating slightly above 100 kDa, which is consistent with the predicted molecular weights of known NLRP3 isoforms, was detected in the control-transfected cells and was reduced by 77% using siNLRP3 (Figure 4B) (Kummer et al., 2007). The identity of this band as NLRP3 was further confirmed using a lysate of HEK293T cells overexpressing NLRP3 tagged with DDK (identical to

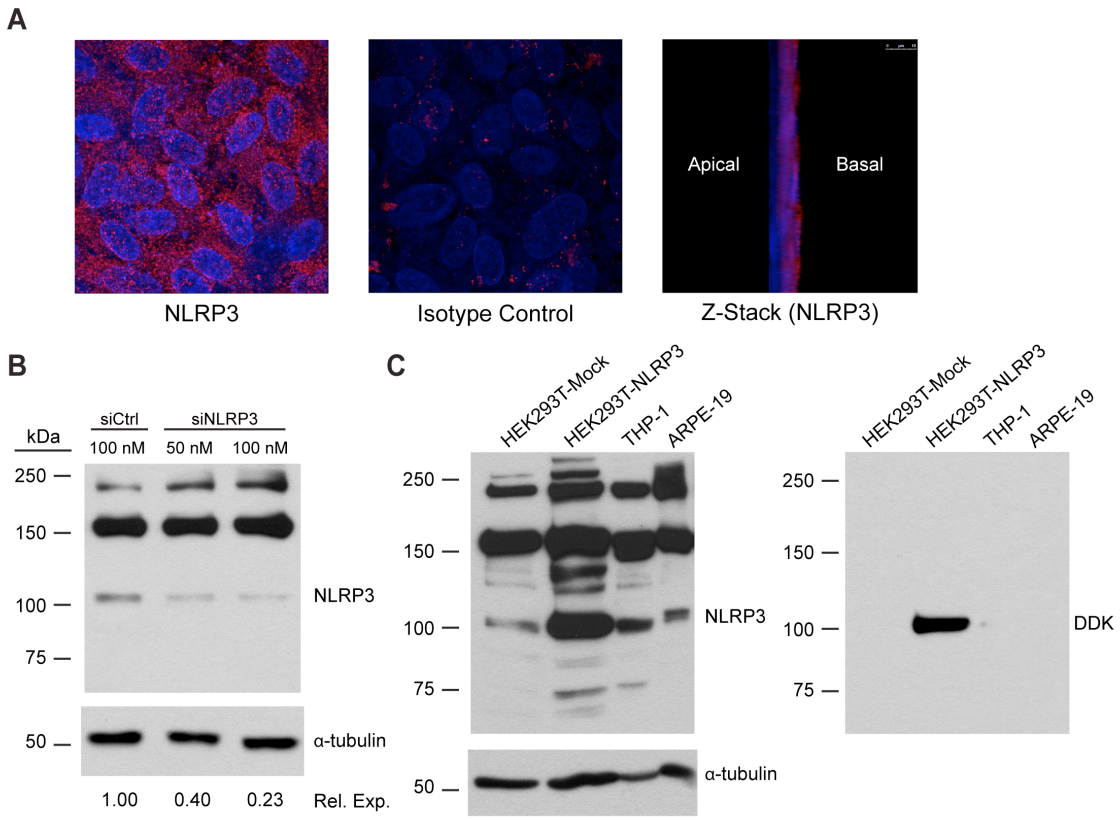

Figure 4. Expression of NLRP3 in human RPE cells in vitro. (A) ARPE-19 cells were cultured on Transwell membranes for four weeks to form a polarized monolayer. Monolayers were fixed, permeabilized, and stained for NLRP3 (red) and with DAPI to reveal nuclei (blue). Z-stacks of polarized monolayers were generated using confocal microscopy. (B) ARPE-19 cells grown on plastic wells were transfected with an siRNA pool against human NLRP3 or a control siRNA pool. ARPE-19 lysates were subjected to immunoblotting for NLRP3, with α-tubulin as a loading control. Densitometry was performed using ImageJ (NIH). Tubulin-normalized NLRP3 band densities are presented as their value relative to the control. (C) The lysates of ARPE-19 cells were compared to those of HEK293T cells overexpressing DDK-tagged NLRP3 (HEK293T-NLRP3) and a mock-transfected HEK293T lysate (HEK293T-Mock) to confirm the identify of the NLRP3 band. THP-1 cells served as a reference for NLRP3 levels in a cell type with known NLRP3 inflammasome activity. Lysates were immunoblotted for NLRP3, with α-tubulin as a loading control, and reprobed for the DDK tag fused to the NLRP3 overexpressed in HEK293T cells.

Table 1. Target sequences of siRNAs used in this study. Pooled siRNA against NLRP3 and a non-targeting control siRNA pool were obtained from Dharmacon. Each pool contains 4 siRNAs, whose target sequences are presented above.

siRNA Pool	Target Sequences
NLRP3	#1 – 5'-GCAAGACCAAGACGUGUGA-3' #2 – 5'-GAAGUGGGGUUCAGAUAAU-3' #3 – 5'-UGCAAGAUCUCUCAGCAAA-3' #4 – 5'-GGAUCAAACUACUCUGUGA-3'
Control	#1 – 5'-UGGUUUACAUGUCGACUAA-3' #2 – 5'-UGGUUUACAUGUUGUGUGA-3' #3 – 5'-UGGUUUACAUGUUUUCUGA-3' #4 – 5'-UGGUUUACAUGUUUUCCUA-3'

FLAG). The ~100 kDa band observed in lysates of ARPE-19 co-migrated with a protein that was heavily enriched in the NLRP3-overexpressing lysate compared to mock-transfected HEK293T lysate (Figure 4C). A band at this molecular weight is also present in the human monocytic leukemia cell line THP-1, which has been demonstrated to express NLRP3 and is known to have NLRP3 inflammasome activity (Kanneganti, 2010; Niemi et al., 2011; Rajamaki et al., 2010). Reprobing the same blot with an anti-DDK tag antibody revealed that the enriched band in the NLRP3-overexpressing HEK293T lysate is indeed NLRP3. Taken together, these results conclusively demonstrate the expression of NLRP3 by ARPE-19 cells.

Priming of RPE cells with NF-κB agonists induces pro-IL-1β expression

I next sought to determine if ARPE-19 cells could be primed to express pro-IL-1β, a classical inflammasome substrate. In myeloid cells, pro-IL-1β expression is induced by priming with a NF-κB activating stimulus such as LPS (Hiscott et al., 1993; Schroder and Tschopp, 2010). To determine if NF-κB agonists prime ARPE-19 cells, the cells were treated with LPS, IL-1α, or TNFα at concentrations of 4 ng/ml and 50 ng/ml for 24 or 48 hours. Immunoblotting of ARPE-19 lysates revealed that while pro-IL-1β was barely detectable in the absence of NF-κB agonists, treatment with IL-1α or TNFα induced pro-IL-1β expression, with IL-1α inducing the highest levels (Figure 5A). In contrast, LPS did not upregulate pro-IL-1β under the conditions tested, an observation that is consistent with the fact that the levels of TLR4 are markedly reduced in ARPE-19 compared to primary RPE (Gnana-Prakasam et al., 2008).

Due to the extent of its priming activity, IL-1α was selected for use in subsequent experiments. Thus, I further evaluated the dose- and time-dependency of the induction of pro-IL-1β by IL-1α. Confluent ARPE-19 cells were treated with an IL-1α dose curve for 48 hours (Figure 5B) or with 4 ng/ml IL-1α for a range of times up to 48 hours (Figure 5C). Pro-IL-1β was induced by IL-1α in a dose-dependent manner. Detectable levels of pro-IL-1β were expressed in

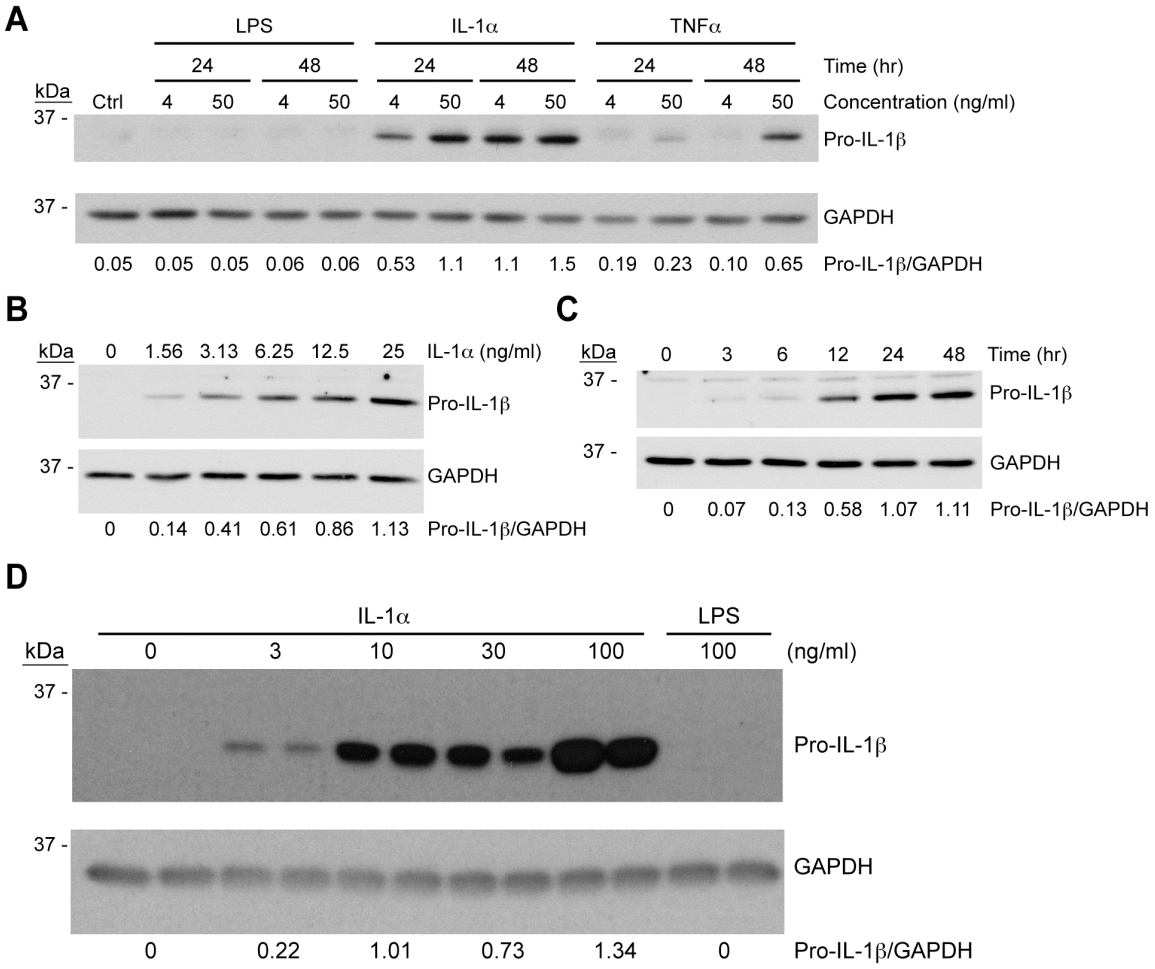

Figure 5. Priming of RPE cells by inducers of NF-κB. (**A**) ARPE-19 cells grown to confluence on plastic wells were treated with the NF-κB activating agents LPS, IL-1α, or TNFα at a concentration of 4 ng/ml or 50 ng/ml for 24 hr or 48 hr, or were left untreated as a negative control (Ctrl). (**B** and **C**) Confluent ARPE-19 cells were treated with increasing concentrations of IL-1α for 48 hr (**B**) or 4 ng/ml IL-1α for increasing lengths of time (**C**). (**D**) hfRPE cells were treated in duplicate with a range of concentrations of IL-1α, or 100 ng/ml LPS, for 24 hr. Cell lysates were immunoblotted for pro-IL-1β, after which blots were stripped and reprobed for GAPDH as a loading control. Densitometry was performed using ImageJ (NIH), and GAPDH-normalized band intensities are displayed for each condition.

response to the lowest dose of IL-1α evaluated, approximately 1.5 ng/ml. Doubling the IL-1α dose to roughly 3.1 ng/ml resulted in a nearly threefold increase in pro-IL-1β levels. Each additional twofold increase in IL-1α dose, up to 25 ng/ml, the maximum dose tested, induced a 1.3- to 1.5-fold upregulation in pro-IL-1β. Treatment of ARPE-19 cells with 4 ng/ml IL-1α upregulated pro-IL-1β expression in a time-dependent fashion. At three hours, pro-IL-1β was barely detectable, and this level roughly doubled by six hours. Between six and twelve hours, pro-IL-1β levels increased by approximately 4.5-fold. From twelve to 24 hours, pro-IL-1β was further upregulated by 1.8-fold, at which point levels of pro-IL-1β reached a maximum, changing minimally between 24 and 48 hours.

After observing that molecules such as IL-1α can prime ARPE-19 cells to express pro-IL-1β, the effect of IL-1α on primary human fetal RPE (hfRPE) cells was evaluated. hfRPE cells were treated with a dose curve of IL-1α up to 100 ng/ml (Figure 5D); other hfRPE cells were treated with 100 ng/ml LPS as a comparison. As expected, pro-IL-1β was not expressed by unprimed hfRPE cells but, similar to ARPE-19 cells, the addition of IL-1α led to a dose-dependent increase in the expression of pro-IL-1β. LPS did not induce pro-IL-1β in hfRPE cells.

Expression of NLRP3 inflammasome components in human RPE cells

ARPE-19 cells were next evaluated for the expression of the critical NLRP3 inflammasome components ASC and procaspase-1. In addition, I examined the effect of priming on the expression of NLRP3, ASC, and procaspase-1. In addition to inducing the expression of pro-IL-1β, priming has also been reported to increase the expression of other inflammasome components, including NLRP3, in some cell types such as mouse macrophages (Bauernfeind et al., 2009). ARPE-19 cells were treated with a dose-curve of IL-1α up to 25 ng/ml, and lysates were immunoblotted for NLRP3, ASC and caspase-1.

NLRP3 was expressed by ARPE-19 cells in the absence of priming, and its expression

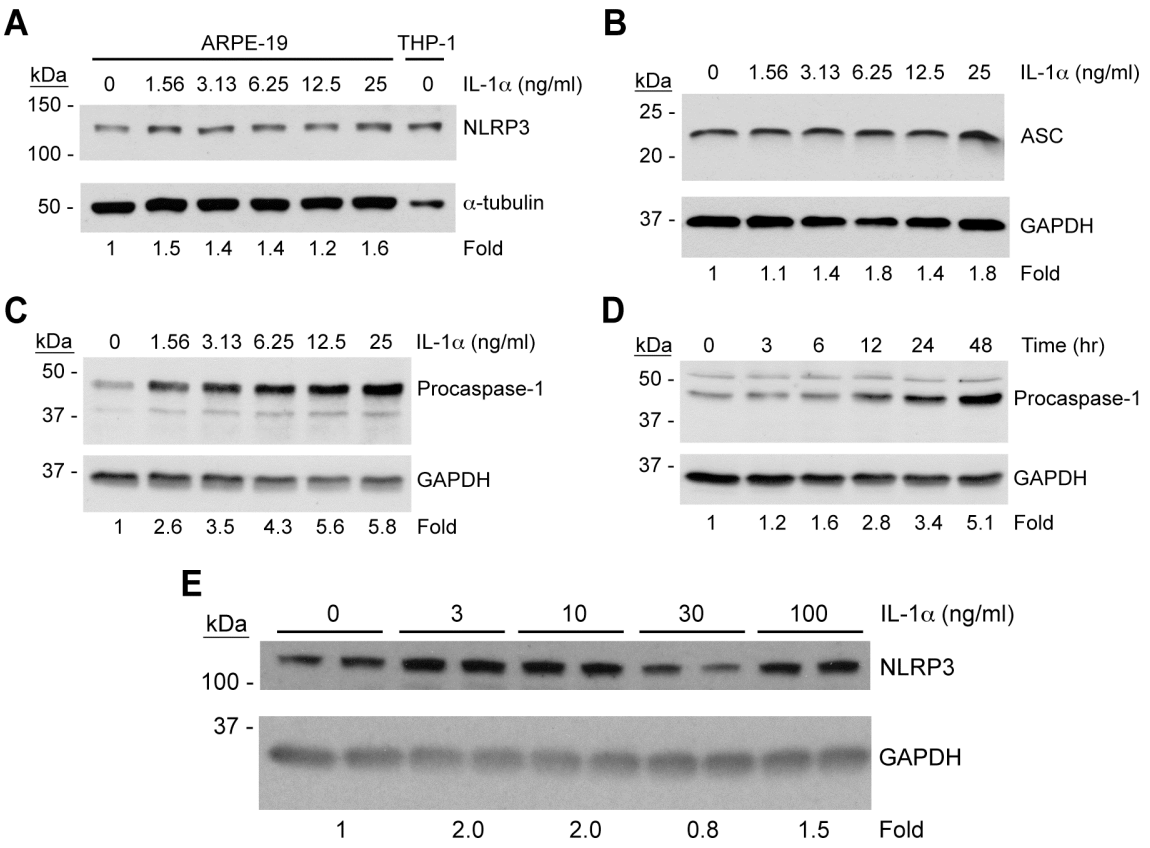

Figure 6. Effect of priming on expression of NLRP3 inflammasome components in human RPE cells. ARPE-19 cells were treated with (A, B, and C) increasing concentrations of IL-1α for 48 hours or (D) with 4 ng/ml IL-1α for a time course up to 48 hours. Lysates were immunoblotted for (A) NLRP3, (B) ASC, or (C and D) procaspase-1. (E) hfRPE cells were treated in duplicate with a range of concentrations of IL-1α for 24 hours, and lysates were immunoblotted for NLRP3. All blots were stripped and reprobed for GAPDH or α-tubulin as a loading control. Densitometry was performed using ImageJ, and band intensities were normalized to GAPDH or α-tubulin. Densitometry results are expressed as a fold-change compared to unprimed cells.

was largely unresponsive to IL-1α (Figure 6A). Maximal NLRP3 induction of 1.6-fold was achieved at 25 ng/ml IL-1α, the highest IL-1α dose tested (Figure 6A). Similarly, ASC was expressed under basal conditions and was not increased by treatment with IL-1α (Figure 6B). On the other hand, procaspase-1 was expressed under unprimed conditions but its levels were further increased by IL-1α in a dose-dependent manner, reaching a maximal induction of five- to six-fold when stimulated by 12.5 ng/ml IL-1α or higher (Figure 6C). The time-dependency of procaspase-1 upregulation was evaluated by treating ARPE-19 cells with 4 ng/ml IL-1α for a range of times up to 48 hours. Procaspase-1 levels increased throughout the time course, reaching a five-fold induction at 48 hours (Figure 6D). The effect of priming on NLRP3 expression was also assessed in primary hfRPE cells (Figure 6E). Like ARPE-19 cells, hfRPE cells expressed NLRP3 under basal conditions, and the levels of NLRP3 were not further increased by treatment with IL-1α.

DISCUSSION

These data establish an association between characteristics of human AMD and NLRP3 expression by RPE. I detected NLRP3 protein in the RPE of donor human eyes affected by AMD, but not in eyes of age-matched controls. NLRP3 expression in vivo was associated with both GA and neovascular AMD, and was detected both intracellularly in the RPE as well as extracellularly in drusen and in the vicinity of BrM, likely released from dying RPE cells. These results also demonstrate the expression of NLRP3 inflammasome components by RPE cells in vitro and the induction of the IL-1β precursor, pro-IL-1β, by RPE cells. Studies in myeloid cells have shown that NF-κB-mediated priming signals induce the expression of both NLRP3 and pro-IL-1β (Bauernfeind et al., 2009), and we speculate that molecular changes in the outer retina that occur during the pathogenesis of AMD, such as the accumulation of proinflammatory molecules in drusen deposits (Ambati et al., 2013), may induce these changes in RPE.

It is unclear whether the association of NLRP3 expression with GA and neovascular AMD reflects shared events in their pathogeneses or are a common result of distinct processes, as their etiologies are currently unsolved. Dry AMD can lead to neovascular AMD (Imrie and Bailey, 2007), suggesting common events in their development. However, some cases of neovascular AMD occur in the absence of dry AMD, and it is hypothesized that neovascular AMD and GA have differing etiologies, with CC degeneration and drusen-mediated RPE atrophy proposed as their initial insults, respectively (Bhutto and Lutty, 2012). However, CC loss may occur secondary to RPE deterioration, and vice versa, so neovascular AMD and GA may share common pathological features, but dissimilar initial insults. It is likely that NLRP3 upregulation is induced by one or more of these shared characteristics.

Unlike the pigment epithelium in vivo, RPE cells in vitro expressed NLRP3 without proinflammatory stimulation. This is likely due to culture conditions where cells are released from contact inhibition in the process of isolation and subsequent trypsinization and are continuously exposed to elevated levels of stimulators present in the serum. The unstimulated ARPE-19 and hfRPE cells, however, did not express pro-IL-1β, indicating that culture conditions do not entirely mimic conditions of priming. Priming ARPE-19 or hfRPE cells with IL-1α induced pro-IL-1β expression. The finding that LPS did not induce pro-IL-1β expression in ARPE-19 cells is consistent with previous reports that whereas primary RPE cells express the LPS receptor TLR4, it is downregulated in ARPE-19 cells (Gnana-Prakasam et al., 2008; Kindzelskii et al., 2004; Kumar et al., 2004). However, the inability of LPS to prime sixth- to eighth-passage hfRPE cells suggests that expression of TLR4 or a downstream signaling molecule may decline with passage in culture.

The identity of the molecule(s) that might prime the RPE in the context of AMD is not known. As the expression of pro-IL-1β is downstream of NF-κB, any molecule that activates NF-κB is a potential priming agent (Hiscott et al., 1993; Schroder and Tschopp, 2010). Although IL-1α was the most potent priming agent evaluated in this study, I also found that TNFα induced

RPE cells to express pro-IL-1β. TNFα, which has been localized in choroidal neovascular membranes obtained from AMD-affected eyes (Oh et al., 1999; Wang et al., 1999), regulates several activities in RPE cells, such as attachment, spreading, and migration (Jin et al., 2000; Yang et al., 2005), and has been implicated in the development of laser-induced CNV (Jasielska et al., 2010; Lichtlen et al., 2010). Additionally, higher levels of proteins adducted to CEP have been detected in eyes from donors with AMD than in age-matched controls (Hollyfield et al., 2008). CEP-adducted human serum albumin (HSA) has been shown to prime murine and human macrophages and mononuclear cells (Doyle et al., 2012), so it is plausible that CEP adducts act similarly on RPE cells. Thus, although the specific stimuli that prime RPE cells in AMD remain uncertain, it is clear that NLRP3 is upregulated in the RPE of eyes afflicted with AMD. Thus, one or more of the molecular changes in the outer retina that are associated with AMD prime the NLRP3 inflammasome in human RPE cells.

CAVEATS, LIMITATIONS, AND POTENTIAL RESOLUTIONS

It is important to note a caveat associated with the immunohistochemistry of human ocular sections described in this chapter, which detected NLRP3 in the RPE of eyes affected by advanced AMD (Figure 3). The antibody used for NLRP3 immunohistochemistry is the same antibody that was used for NLRP3 immunoblotting (Figure 4), where it can be seen that it yielded a number of prominent non-specific bands. It is the case that the same antisera did not yield any labeling in the sections of retinas from donor eyes without AMD. However, to be entirely confident in the staining, it would be important to verify the specificity of the immunohistochemistry. An appropriate control to assess antibody specificity would be to stain with antisera that had been immunoabsorbed with the antibody's immunizing peptide. Use of immunoabsorbed antisera would eliminate specific staining and any labeling observed would be deemed non-specific.

CHAPTER 3

ACTIVATION OF THE NLRP3 INFLAMMASOME IN RPE CELLS VIA LYSOSOMAL DESTABILIZATION

PREFACE

The Results and Discussion sections of this chapter are adapted from portions of the article, "NLRP3 inflammasome activation in retinal pigment epithelial cells by lysosomal destabilization: implications for age-related macular degeneration," by W.A. Tseng, T. Thein, K. Kinnunen, K. Lashkari, M.S. Gregory, P.A. D'Amore, and B.R. Ksander in *Investigative Ophthalmology & Visual Science*, January 2013, Vol. 54, No. 1, pages 110-120, the copyright of which is held by The Association for Research in Vision and Ophthalmology, Inc. Figures 8 and 10 have been added to include data on hfRPE cells and the text of the Results and Discussion sections have been adapted to reflect this new data.

RATIONALE

NLRP3 inflammasome activity is regulated at two levels by signals associated with cellular danger, such as pathogen infection or tissue damage (Rathinam et al., 2012a). Certain signals induce the activation of the NLRP3 scaffold protein, resulting in inflammasome assembly and activation of caspase-1, whereas other signals are responsible for the transcriptional induction of pro-IL-1β, in a process known as priming. In many cell types, priming signals are also necessary for the expression of NLRP3 (Bauernfeind et al., 2009). I have demonstrated that RPE cells express all of the components of the inflammasome: NLRP3, ASC, and procaspase-1. I have also shown that priming RPE cells with NF-κB agonists such as IL-1α induces expression of pro-IL-1β. However, it remains to be seen whether the NLRP3 inflammasome can be activated in RPE cells. The danger signals that activate the NLRP3 inflammasome are numerous and chemically diverse, and include extracellular ATP, microbial pore-forming toxins, and a wide variety of crystals and insoluble particles or aggregates (Franchi et al., 2012). The crystals and insoluble substances that have been demonstrated to induce the NLRP3 inflammasome include Aβ, asbestos fibers, and crystals of MSU, calcium pyrophosphate dihydrate, silica, cholesterol, or alum.

Crystalline or insoluble matter has been reported to induce the NLRP3 inflammasome via a mechanism involving lysosomal destabilization (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008). Evidence suggests a model in which crystals and particles are internalized into endosomes or phagosomes, which fuse with lysosomes. Crystals and particulate matter damage the endolysosomal or phagolysosomal membrane, leading to the leakage of lysosomal enzymes such as cathepsins into the cytosol where they mediate signaling that activates NLRP3. The mechanism through which NLRP3 is activated remains unclear, but may involve degradation of an inhibitory NLR, NLRP10 (Murphy et al., 2013). In support of this model of mechanical destabilization of the lysosomes, blockade of phagocytosis

by disrupting cytoskeletal dynamics abrogates crystal-induced inflammasome activation (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008). Similarly, preventing the activity of pH-sensitive lysosomal enzymes by inhibiting lysosomal acidification suppresses crystal-mediated NLRP3 inflammasome induction. In addition, activation of the NLRP3 inflammasome by crystals is partially but significantly inhibited by genetic deletion or pharmacological blockade of cathepsin B or cathepsin L. Furthermore, phagocytosis-independent disruption of lysosomes using the lysosomotropic compound Leu-Leu-OMe also induces the NLRP3 inflammasome via lysosomal enzymes including cathepsin B.

During AMD pathogenesis, RPE cells are exposed to insoluble material in the form of drusen deposits that accumulate between the basement membrane of the RPE and the inner collagenous layer of BrM. Drusen have a heterogeneous composition and are comprised of lipids such as cholesterol and proteins such as vitronectin, complement proteins, and amyloid proteins (Hageman et al., 1999; Isas et al., 2010; Wang et al., 2010). Phagocytosis of the drusen components Aβ and complement protein C1Q by myeloid-derived cells induces the NLRP3 inflammasome via lysosomal destabilization (Doyle et al., 2012; Halle et al., 2008). Furthermore, incubation of myeloid cells with drusen isolated from AMD-affected eyes activates the NLRP3 inflammasome (Doyle et al., 2012). In addition to drusen deposition, AMD pathogenesis also involves the accumulation of a pigmented, granular substance called lipofuscin in RPE lysosomes. RPE lipofuscin is believed to be the product of incomplete degradation of phagocytosed material. A2E, a constituent of RPE lipofuscin, has detergent-like properties and has been demonstrated to disrupt lysosomal membranes (Schutt et al., 2002; Sparrow et al., 2006).

In light of the involvement of lysosomal disruption in NLRP3 inflammasome activation in myeloid-derived cells, the accumulation of potential sources of RPE lysosomal damage during AMD progression, and the presence of NLRP3 inflammasome components in RPE cells, I hypothesize that lysosomal destabilization induces the NLRP3 inflammasome in RPE cells. This

chapter presents evidence demonstrating that chemical disruption of RPE lysosomes triggers inflammasome activation, as evidenced by the activation of caspase-1 and the induction of inflammasome effector functions such as IL-1β processing and caspase-1-dependent programmed cell death, known as pyroptosis.

RESULTS

Lysosomal destabilization in RPE cells activates caspase-1

Having found that ARPE-19 cells expressed the components of the NLRP3 inflammasome (Chapter 2), I assessed whether the destabilization of their lysosomes would lead to inflammasome activation as measured by the activation of caspase-1, and I evaluated the involvement of lysosomal enzymes in this process. The lysosomotropic agent Leu-Leu-OMe, which is converted within the lysosome to membranolytic derivatives by dipeptidyl peptidase I (DPP-I), was used to disrupt lysosomes (Thiele and Lipsky, 1990). Staining with acridine orange, which fluorescently labels DNA and RNA as green and lysosomes as red, was used to assess the effects of Leu-Leu-OMe on lysosomal integrity (Figure 7A). Whereas control ARPE-19 cells contained red punctate structures characteristic of intact lysosomes, treatment with 1 mM Leu-Leu-OMe triggered a loss of lysosomal staining indicative of lysosomal destabilization.

Caspase-1 activation was evaluated using the fluorescent labeled inhibitor of caspases (FLICA) probe FAM-YVAD-FMK that specifically labels active caspase-1. While activated caspase-1 was undetectable in untreated ARPE-19 cells, treatment with 1 mM Leu-Leu-OMe for 2 hours induced significant caspase-1 activation (Figure 7, B and C). To control for the possibility that effects of Leu-Leu-OMe unrelated to lysosomal destabilization were responsible for inflammasome activation, a DPP-I inhibitor (Gly-Phe-CHN_2) was used to block the disruption of lysosomes by Leu-Leu-OMe. Addition of Gly-Phe-CHN_2 30 minutes prior to Leu-Leu-OMe

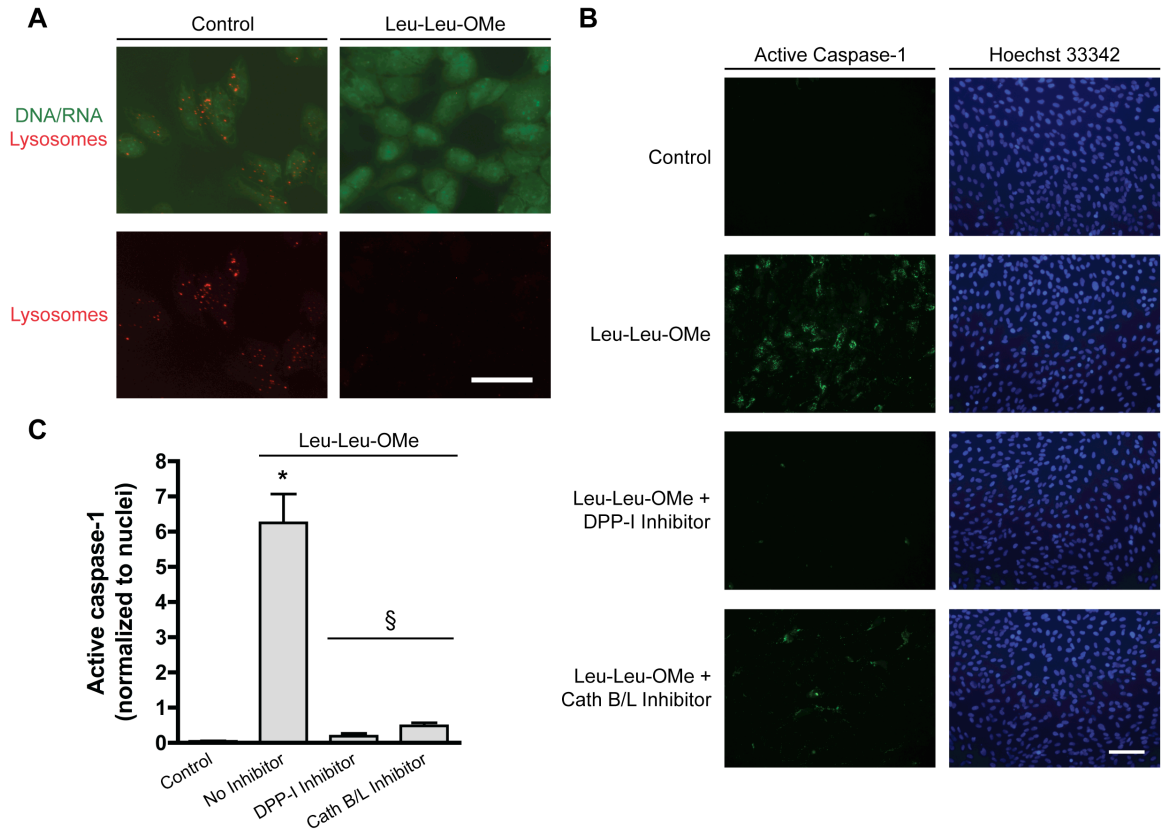

Figure 7. Lysosomal destabilization activates caspase-1 in ARPE-19 cells. (A) ARPE-19 cells were stained with 5 μM acridine orange for 30 minutes and treated for 30-45 minutes with 1 mM Leu-Leu-OMe or control buffer. Fluorescence microscopy was used to detect acridine orange sequestered in lysosomes (red) or bound to DNA or RNA (green). Scale bars, 50 μm. (B) ARPE-19 cells were primed with 4 ng/ml IL-1α for 48 hours. Cells were treated with 1 mM Leu-Leu-OMe for 2 hours to disrupt lysosomes, or left untreated (Control). Lysosomal destabilization induced by Leu-Leu-OMe was blocked by inhibiting DPP-I via addition of 10 μM Gly-Phe-CHN_2 to cells 30 minutes before addition of Leu-Leu-OMe. The lysosomal proteases cathepsins B and L were inhibited using 50 μM Z-FF-FMK. Active caspase-1 was detected by the FLICA probe FAM-YVAD-FMK (green). Nuclei were labeled by staining with Hoechst 33342 (blue). Scale bars, 100 μm. (C) Green active caspase-1 signal was quantified and normalized to number of nuclei. Numerical data are represented as mean ± standard error of the mean (SEM); n = 3. * indicates $P<0.01$ vs. Control; § indicates $P<0.01$ vs. Leu-Leu-OMe with no inhibitor.

abrogated its ability to activate the inflammasome (Figure 7, B and C), supporting the conclusion that lysosomal destabilization triggers the NLRP3 inflammasome in ARPE-19 cells.

Previous reports demonstrate that lysosomal enzymes, particularly cathepsins B and L, released from destabilized lysosomes play a critical role in the induction of the NLRP3 inflammasome in myeloid cells (Duewell et al., 2010; Hornung et al., 2008; Rajamaki et al., 2010). Therefore, the cathepsin B and L inhibitor Z-FF-FMK was used to assess the involvement of these lysosomal enzymes in NLRP3 activation in ARPE-19 cells (Iwata et al., 2003; Ravanko et al., 2004). Pre-treatment with Z-FF-FMK significantly inhibited activation of caspase-1 by Leu-Leu-OMe (Figure 7, B and C), indicating a role for cathepsins B and/or L in NLRP3 inflammasome induction in RPE cells.

After the demonstration that lysosomal disruption activated caspase-1 in ARPE-19 cells via a cathepsin-mediated mechanism, I evaluated inflammasome activity in primary hfRPE cells using the caspase-1 FLICA probe (Figure 8). The effects of priming and lysosomal destabilization on caspase-1 activation were assessed in hfRPE cells cultured on plastic (Figure 8A) or maintained on Transwell membranes to induce polarization and maturation (Figure 8B). In the absence of lysosomal damage, no active caspase-1 was detected. However, disruption of lysosomal integrity with Leu-Leu-OMe triggered caspase-1 activation. Without priming, only minimal levels of active caspase-1 were observed, but substantially higher levels were generated in cells that were primed for 24 hours with IL-1α prior to Leu-Leu-OMe treatment. These results were similar whether hfRPE cells were maintained on Transwell membranes or grown on plastic. Furthermore, inhibition of DPP-I in order to prevent Leu-Leu-OMe-mediated lysosomal membrane disruption abrogated caspase-1 activation (Figure 8C). Inhibition of cathepsins B and L or cathepsin B alone also blocked caspase-1 activation in hfRPE cells by lysosomal destabilization (Figure 8C).

Lysosomal destabilization in RPE cells induces IL-1β release and cytotoxicity

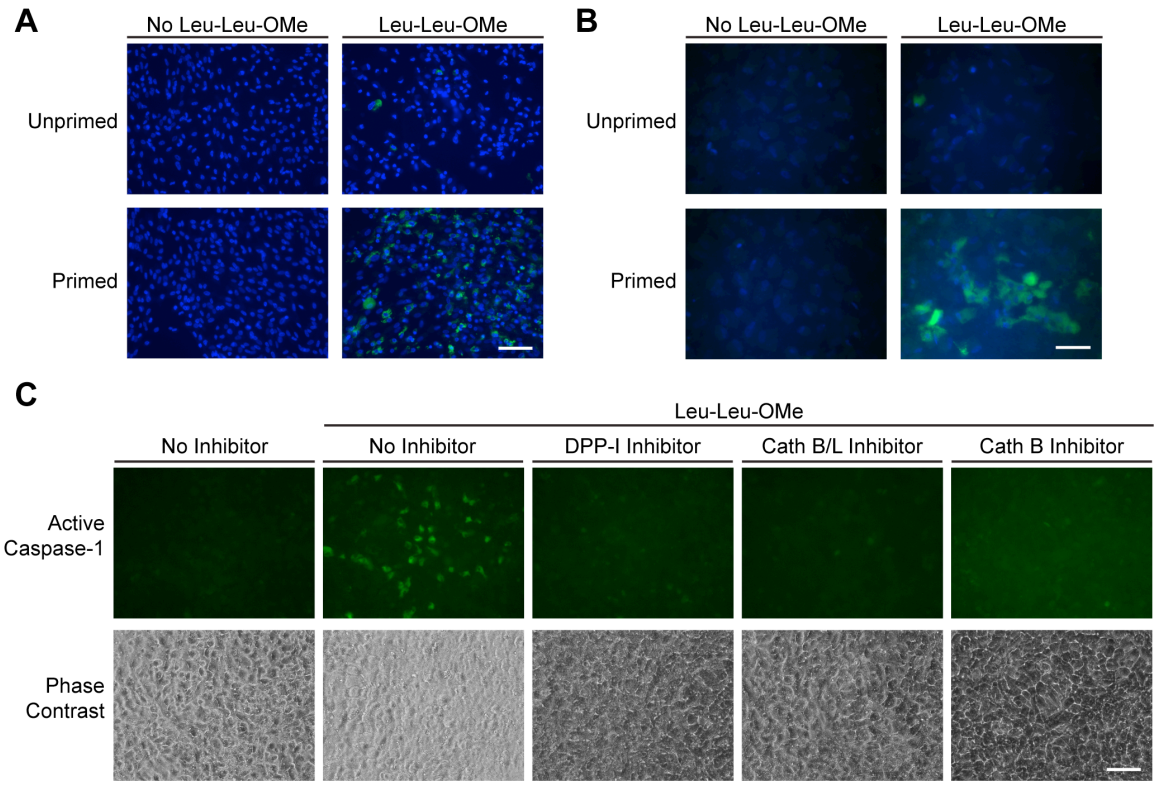

Figure 8. Lysosomal disruption activates caspase-1 in primary RPE cells. (A) hfRPE cells were grown to confluence on plastic wells or (B) maintained on Transwell membranes and were primed with 10 ng/ml IL-1α or treated with control buffer for 24 hours. Cells were then treated with 1 mM Leu-Leu-OMe or control buffer for 2 hours. Active caspase-1 (green) was detected with the FLICA probe FAM-YVAD-FMK, and Hoechst 33342 was used to stain nuclei (blue). (C) hfRPE cells maintained on Transwell membranes were primed with 10 ng/ml IL-1α for 24 hours. Appropriate wells were then treated with the DPP-I inhibitor Gly-Phe-CHN_2 (10 μM), the cathepsin B and L inhibitor Z-FF-FMK (50 μM) and the selective cathepsin B inhibitor CA-074-Me (50 μM). After 30 minutes, lysosomal disruption was induced by treatment with 1 mM Leu-Leu-OMe for 2 hours. Active caspase-1 (green) was detected with FAM-YVAD-FMK, and phase contrast microscopy was used to image cell morphology. Scale bars: (A) 100 μm, (B) 50 μm, (C) 100 μm.

I next investigated whether lysosomal disruption can induce release of mature IL-1β from primed cells. Although IL-1β ELISAs are much less sensitive for pro-IL-1β than the mature form (Herzyk et al., 1992), pro-IL-1β released from dying cells may be misinterpreted as low levels of cleaved IL-1β. Immunoblotting demonstrated that Leu-Leu-OMe triggered the release of mature IL-1β from IL-1α-primed ARPE-19 cells that co-migrated with recombinant human mature IL-1β (Figure 9A). Thus, specific detection of the mature form of IL-1β can only be achieved by immunoblotting of conditioned media, as it clearly distinguishes between mature IL-1β (17 kDa) and its precursor (31-35 kDa) by virtue of their molecular weights.

After demonstrating that mature IL-1β is secreted by ARPE-19 cells treated with Leu-Leu-OMe, I used ELISA to quantify the amount of IL-1β secreted and to investigate the mechanisms involved. Leu-Leu-OMe induced the release of approximately 40 pg/ml IL-1β after 3 hours and this release was completely blocked by DPP-I inhibition, indicating that Leu-Leu-OMe acted through lysosomal destabilization (Figure 9B). The selective caspase-1 inhibitor Z-YVAD-FMK also significantly reduced IL-1β secretion, indicating that the lysosomal damage-induced IL-1β release from ARPE-19 cells is mediated by caspase-1 and the inflammasome. Additionally, inhibition of cathepsins B and L using Z-FF-FMK blocked IL-1β secretion, supporting a role for these lysosomal enzymes in inflammasome activation in ARPE-19 cells.

Treatment with Leu-Leu-OMe also caused substantial cytotoxicity, as assessed by lactate dehydrogenase (LDH) release. ARPE-19 cells treated with Leu-Leu-OMe for 3 hours exhibited 40-50% cell death (Figure 9B). As with IL-1β secretion, cytotoxicity induced by Leu-Leu-OMe was completely blocked by inhibition of DPP-I or cathepsins B and L. Furthermore, the cell death caused by lysosomal disruption was completely suppressed by the caspase-1 inhibitor, indicating an inflammasome-mediated death mechanism.

Inflammasome-mediated IL-1β release (Figure 10A) and cell death (Figure 10B) were also evaluated in primary hfRPE cells. IL-1α-primed hfRPE cells treated with Leu-Leu-OMe released more than 60 pg/ml of IL-1β, as measured by ELISA. Consistent with the lack of pro-

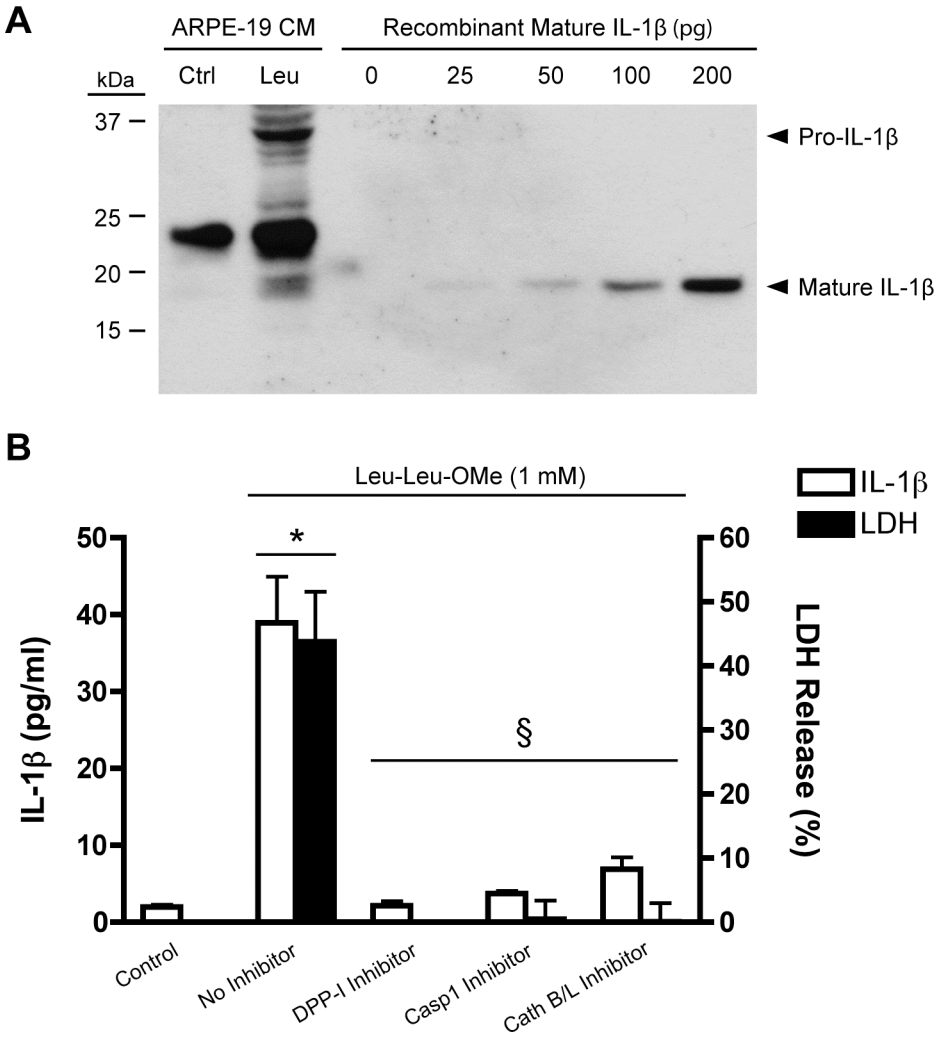

Figure 9. ARPE-19 cells secrete IL-1β and undergo pyroptotic cell death in response to lysosomal destabilization. (A) ARPE-19 cells primed with 15 ng/ml IL-1α for 48 hr were treated with 1 mM Leu-Leu-OMe (Leu) to disrupt lysosomes or received control buffer (Ctrl). After 3 hours, conditioned media (CM) were concentrated and immunoblotted to detect mature IL-1β (17 kDa) and distinguish it from its precursor (31-35 kDa). A serial dilution of 0-200 pg recombinant human IL-1β was used as reference. (B) IL-1β ELISA was performed on CM from ARPE-19 cells treated with 1 mM Leu-Leu-OMe. LDH release was quantified to evaluate cytotoxicity. The selective inhibitors Gly-Phe-CHN_2 (5 μM), Z-YVAD-FMK (10 μM), and Z-FF-FMK (50 μM) were used to block the activity of DPP-I, caspase-1, and cathepsins B and L, respectively. Data represent mean ± SEM of three experiments. * indicates $P<0.01$ vs. Control, § indicates $P<0.01$ vs. Leu-Leu-OMe with no inhibitor.

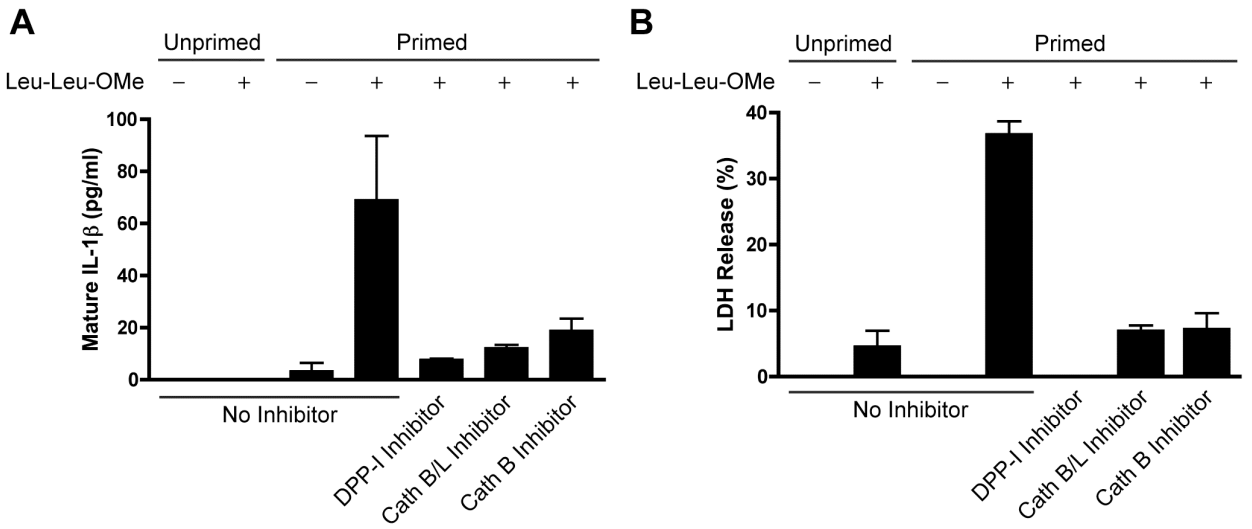

Figure 10. Lysosomal destabilization induces IL-1β release and pyroptosis in human primary RPE cells. hfRPE cells were primed with 10 ng/ml IL-1α or treated with control buffer for 24 hours. The DPP-I inhibitor Gly-Phe-CHN_2 (10 μM), the caspase-1 inhibitor Z-YVAD-FMK (10 μM), the cathepsin B and L inhibitor Z-FF-FMK (50 μM), and the selective cathepsin B inhibitor CA-074-Me (50 μM) were added to appropriate wells. After 30 minutes, 1 mM Leu-Leu-OMe was added to destabilize lysosomes. Following a 3-hour incubation, conditioned media were collected. (A) ELISA was performed to quantify levels of IL-1β released into hfRPE conditioned media. (B) LDH levels in hfRPE conditioned media were quantified to evaluate cytotoxicity. Experiment was performed in triplicate, and data are represented as the mean ± standard deviation.

IL-1β expression in RPE cells under basal conditions, unprimed hfRPE cells did not release IL-1β even when treated with Leu-Leu-OMe. Primed cells treated with Leu-Leu-OMe also exhibited 30-40% cytotoxicity, as detected by LDH release. Cell death was significantly lower in unprimed hfRPE cells compared to primed cells. Lysosomal destabilization was required for both IL-1β release and cell death, as they were abrogated by inhibition of DPP-I. Blockade of cathepsins B and L significantly reduced IL-1β release and cytotoxicity, and inhibition of cathepsin B alone lowered IL-1β release and cell death to a similar degree, suggesting that cathepsin B is the predominant lysosomal enzyme involved in inflammasome activation in primary RPE cells.

DISCUSSION

Chemical disruption of lysosomes using Leu-Leu-OMe triggered inflammasome activation in RPE cells, as evidenced by the detection of active caspase-1, which mediated IL-1β release and RPE cell death. Pre-treatment with Gly-Phe-CHN_2 inhibited the intralysosomal conversion of Leu-Leu-OMe to membranolytic metabolites by DPP-I, preventing Leu-Leu-OMe-mediated lysosomal destabilization and inflammasome activation. Blocking lysosomal destabilization abolished caspase-1 activation, IL-1β release, and RPE cytotoxicity. These results demonstrate that inflammasome activation and its downstream effects were induced by lysosomal destabilization, rather than unrelated effects of Leu-Leu-OMe. Additionally, the fact that inhibition of the lysosomal enzymes cathepsins B and L blocked lysosomal damage-induced inflammasome activation, IL-1β release, and pyroptosis is consistent with previous reports implicating these enzymes as mediators of NLRP3 inflammasome assembly in myeloid-derived cells (Duewell et al., 2010; Hornung et al., 2008). Whereas cathepsins B and L each played only a partial role in NLRP3 activation in myeloid cells, cathepsin B was responsible for a majority of the caspase-1 activation, IL-1β, and cell death induced by lysosomal destabilization in primary RPE cells, suggesting that cathepsin B is the predominant lysosomal enzyme

responsible for NLRP3 induction in the RPE.

These results support a model in which RPE lysosomal damage results in the leakage of cathepsin B into the cytosol, where it activates NLRP3 through either direct interaction or an intermediary signaling pathway. A recent study reported a mechanism for the induction of NLRP3 inflammasome formation by phagolysosomal destabilization in glial cells, in which cathepsins B and L degrade NLRP10, a NLR that inhibits NLRP3 inflammasome assembly by binding ASC and preventing it from interacting with NLRP3 (Murphy et al., 2013). Therefore, release of cathepsins B and L from lysosomes into the cytosol facilitates NLRP10 degradation and mediates NLRP3 inflammasome activation. It is likely that this mechanism mediates NLRP3 inflammasome formation in RPE cells as well. As expression of NLRP10 has not yet been reported in RPE cells, the possibility exists that another protein performs its function in the RPE.

Intracellular pro-IL-1β has been reported to be produced by RPE cells (Planck et al., 1993), but my finding is the first to demonstrate inflammasome-mediated processing of mature IL-1β from RPE cells. As expected, levels of IL-1β released from RPE cells are significantly lower than those released from myeloid cells in response to lysosomal disruption, which are on the order of 1 ng/ml (Hornung et al., 2008). The term “para-inflammation” has been coined to describe a response to tissue stress that is intermediate between inflammation and the basal state (Medzhitov, 2008). Such low-level inflammation is hypothesized to play a role in age-related and inflammatory disorders such as AMD and atherosclerosis (Buschini et al., 2011; Xu et al., 2009). I speculate that the IL-1β released by RPE cells in response to lysosomal damage mediates para-inflammation, thus contributing to AMD. A number of AMD-associated insults could be responsible for RPE lysosomal destabilization and inflammasome activation during the pathogenesis of AMD. Isolated drusen deposits as well as the drusen components Aβ and complement factor C1Q have been shown to activate the NLRP3 inflammasome in myeloid cells (Doyle et al., 2012; Halle et al., 2008; Isas et al., 2010). Additionally, the formation of drusen is

hypothesized to occur due to exocytosis of undegraded waste material resulting from impaired lysosomal digestive capability (Ambati et al., 2013). Therefore, drusen deposition may be secondary to the accumulation of insoluble substances in lysosomes that could disrupt the lysosomal membrane. Furthermore, the lipofuscin component A2E, which accumulates in RPE lysosomes, has an amphiphilic molecular structure that grants it detergent-like chemical properties that disrupt lysosomal membranes (Schutt et al., 2002; Sparrow et al., 1999).

I demonstrated that RPE cytotoxicity resulting from lysosomal destabilization is caspase-1-dependent. This is characteristic of 'pyroptosis,' a mode of programmed cell death mediated by the inflammasome and caspase-1, rather than apoptotic caspases such as caspase-3 (Miao et al., 2010a). Although apoptosis and pyroptosis are both processes of regulated cell death, pyroptosis is a proinflammatory mode of cell death, whereas apoptosis is non-inflammatory. While the plasma membrane is speculated to remain intact during apoptosis, pyroptosis involves plasma membrane rupture and release of intracellular contents, allowing for its quantification by measurement of LDH release (Bergsbaken et al., 2009; Fernandes-Alnemri et al., 2009; Suzuki et al., 2007). Hence, I speculate that the inflammasome may contribute to AMD via both cytokine release and pyroptotic RPE death.

The data reported here support a two-signal model of NLRP3 inflammasome induction by disruption of RPE lysosomes. Priming signals such as IL-1α, TNFα, or CEP adducts induce expression of pro-IL-1β and NLRP3. Disruption of RPE lysosomes, which may be caused by lipofuscin, drusen components, or other insoluble lysosomal contents, triggers NLRP3 inflammasome assembly via lysosomal enzymes such as cathepsin B. The assembled inflammasome activates caspase-1, which cleaves pro-IL-1β to form mature IL-1β and also mediates pyroptotic RPE cell death. My results have shown that both priming and lysosomal damage must occur for RPE cells to release IL-1β. Furthermore, priming substantially elevates the levels of active caspase-1 generated following lysosomal destabilization, and priming also significantly increases pyroptosis. Although these processes are independent of pro-IL-1β

expression, they are likely enhanced by the upregulation of procaspase-1 induced by priming with IL-1α.

Two recent studies present different hypotheses regarding the function of the NLRP3 inflammasome pathway in AMD. One study using a mouse model of laser-induced CNV suggests that NLRP3 has a protective role in neovascular AMD by inducing IL-18 release from infiltrating macrophages, which in turn reduces VEGF secretion by RPE cells (Doyle et al., 2012), whereas a second study using a mouse model of *Alu* RNA-induced GA suggests that NLRP3 plays a destructive role in dry AMD by inducing IL-18 secretion from RPE cells (Tarallo et al., 2012). Although it is plausible that IL-18 may protect against CNV via inhibition of VEGF production by RPE cells, the laser injury model of CNV is an acute wound-healing model that bears little mechanistic resemblance to neovascular AMD (Marneros, 2013). As previous studies suggest the NLRP3 inflammasome promotes epithelial tissue repair via IL-18 (Dupaul-Chicoine et al., 2010; Zaki et al., 2010), it is likely that the protective effects of IL-18 in the laser-induced CNV model are a reflection of its role in wound healing, rather than AMD. Nevertheless, it is possible that the NLRP3 inflammasome may have distinct roles in wet and dry advanced AMD, or even influence the development of one form over the other.

The data presented in this chapter demonstrate that lysosomal destabilization can activate the NLRP3 inflammasome in RPE cells, inducing the secretion of the potent proinflammatory cytokine IL-1β from primed cells and pyroptosis. These processes may constitute novel mechanisms for AMD pathogenesis. The activators and effectors of the NLRP3 inflammasome are consistent with the phenotype of AMD. Taken together with convincing genetic data that implicate a role for inflammation (Donoso et al., 2006; Gold et al., 2006; Hageman et al., 2005; Nozaki et al., 2006), our findings suggest a mechanism by which insults such as drusen deposition and lipofuscin accumulation can contribute to AMD pathology.

CAVEATS, LIMITATIONS, AND POTENTIAL RESOLUTIONS

The experiments reported in this chapter to examine the biochemical mechanism of inflammasome activation in RPE cells have are limited by the fact that they rely on small molecule or peptide-based inhibitors of enzymes. Such inhibitors may have off-target activities that can confound the analyses of their effects. For example, peptide fluoromethyl ketones, such as the compounds Z-YVAD-FMK and Z-FF-FMK used in this study to inhibit caspase-1 and cathepsins B and L, respectively, can exhibit off-target inhibition via non-specific reaction of the fluoromethyl ketone moiety with active site cysteines of other enzymes. To account for this issue, experiments involving peptide fluoromethyl ketone inhibitors should include a negative control inhibitor. Z-FA-FMK is often used as a specificity control for experiments involving peptide fluoromethyl ketone inhibitors of caspases, as Z-FA-FMK does not specifically target caspases. However, Z-FA-FMK does target cathepsins B and L, which are believed to mediate NLRP3 inflammasome activation. Therefore, Z-FA-FMK is not a suitable control for this system. Instead, one could use an inhibitor of an apoptotic caspase, such as caspase-8, the initiator caspase of the extrinsic apoptotic pathway. Z-IETD-FMK is a peptide fluoromethyl ketone that targets caspase-8, and is not specific for caspase-1 or cathepsins. If off-target activity is responsible for the effects of Z-YVAD-FMK or Z-FF-FMK, then Z-IETD-FMK should produce similar results.

An alternative approach to addressing the issue of inhibitor specificity is to deplete the protein levels of the enzymes of interest via RNA interference. The enzymes caspase-1, cathepsin B, and cathepsin L could be silenced via siRNA or shRNA in order to validate the results generated by the small molecule inhibitors. Furthermore, to ensure that the effects on inflammasome function are due to knockdown of the target gene, RPE cells can be transfected with an RNAi-resistant mutant version of the gene or its ortholog from a different species. Suppression of inflammasome activation or effector functions such as IL-1β processing or pyroptosis induction by the silencing of the enzyme, followed by rescue of inflammasome

activity by the RNAi-resistant mutant, would confirm the role of the enzyme suggested by the inhibitor studies.

CHAPTER 4

GENERAL DISCUSSION

The NLRP3 inflammasome plays a key role in the innate immune system. In response to a variety of signals indicative of microbial infection or tissue damage, the NLRP3 inflammasome mediates the processing and release of inflammatory cytokines such as IL-1β and IL-18. It also promotes a form of pro-inflammatory programmed cell death known as pyroptosis. However, the dysregulation of NLRP3 inflammasome activity can have highly detrimental effects. Pathological NLRP3 activity can occur as a result of gain-of-function mutations, as in the case of the CAPS spectrum of disorders, or due to the accumulation of NLRP3 agonists including insoluble particles, crystals, or aggregates that activate NLRP3 via lysosomal destabilization. Deposition of substances such as MSU crystals, silica crystals, cholesterol crystals, and Aβ, induce NLRP3 inflammasome activity that leads to inflammatory diseases including gout, silicosis, atherosclerosis, and Alzheimer's disease, respectively (Cassel et al., 2008; Duewell et al., 2010; Halle et al., 2008; Martinon et al., 2006; Rajamaki et al., 2010). My research provides evidence that lysosomal disruption in RPE cells activates the NLRP3 inflammasome, suggesting that the NLRP3 inflammasome may also contribute to the pathogenesis of AMD.

Although drusen deposition, the accumulation of lipofuscin in RPE lysosomes, and RPE lysosomal dysfunction have all been associated with AMD, the nature of their involvement in the development of AMD, if any, has remained unclear. Additionally, although mounting evidence indicates that inflammation plays a role in the pathogenesis of AMD, the mechanism underlying this inflammation has not been determined. The finding that RPE lysosomal destabilization activates the NLRP3 inflammasome, resulting in IL-1β release and pyroptotic cell death, points to a mechanism by which features associated with AMD, such as drusen or lipofuscin, may lead to inflammation and cytotoxicity.

In addition to my work, there are several other recent reports describing NLRP3 inflammasome activity in the RPE or in animal models of AMD that corroborate many of my findings (Doyle et al., 2012; Marneros, 2013; Tarallo et al., 2012). Collectively, this body of research sheds important light on the contribution of the NLRP3 inflammasome to AMD, while

raising key questions about the nature and mechanism of that contribution. As there is currently no Food and Drug Administration-approved treatment for GA, the advanced form of dry AMD, this research may open much-needed avenues for therapeutic intervention. Certain disorders mediated by the NLRP3 inflammasome, such as CAPS, are treated using agents that target the IL-1 pathway, suggesting that it is possible that such treatments may be effective against GA.

Expression of NLRP3 inflammasome components and pro-IL-1β in the RPE

I found that NLRP3 is expressed in the RPE of patients with GA and neovascular AMD, but is absent in the RPE of age-matched individuals without AMD. This finding is consistent with observations in myeloid-derived cells, which indicate that NLRP3 is often not constitutively expressed, but instead requires induction known as priming, which also triggers expression of pro-IL-1β (Bauernfeind et al., 2009). Priming can be achieved by a pro-inflammatory stimulus that activates NF-κB (Hiscott et al., 1993). For example, the NF-κB agonist LPS is frequently used to prime myeloid cells in culture. In contrast to RPE in vivo, I found that cultured RPE cells express NLRP3 constitutively, although production of pro-IL-1β required stimulation by a NF-κB-inducing cytokine such as IL-1α or TNFα. The constitutive expression of NLRP3 by RPE in culture, but not in vivo, is likely due to conditions associated with in vitro cell culture such as the presence of serum or culture on a plastic substratum.

Although priming of RPE cells in vitro is achieved by IL-1α and TNFα, and experiments involving myeloid cells often utilize LPS as a priming agent, the factor or factors that induce NLRP3 and pro-IL-1β expression in vivo remain unclear. However, the observation that NLRP3 is expressed in the RPE of eyes affected by advanced AMD, but not control eyes without AMD, suggests that molecular or cellular changes associated with the development of AMD mediate the pathological priming of the RPE. Of the candidate priming agents that I tested, IL-1α was the

most effective. It is possible that IL-1α may be released as a result of age-related damage associated with AMD. IL-1α is a mediator of sterile inflammation, an inflammatory response against stimuli that are not pathogens (Lukens et al., 2012). Cell death, tissue damage, and particles or crystals such as MSU can activate a sterile inflammatory response. Like IL-1β, IL-1α is synthesized as an intracellular precursor lacking a signal peptide. However, whereas pro-IL-1β is inactive, pro-IL-1α is biologically active, and its release from necrotic or pyroptotic cells induces local inflammation (Kim et al., 2013). Furthermore, various inflammatory cytokines, including IL-1α itself, IL-1β, or TNFα, have been shown to stimulate RPE cells to produce the IL-1α precursor (Jaffe et al., 1992). Death of aged RPE cells may cause the release of IL-1α, leading to the priming of RPE cells in the vicinity.

In addition to IL-1α, TNFα also primes RPE cells in vitro. Macrophage-derived TNFα has been reported to contribute to wet AMD, as macrophages present in surgically excised choroidal neovascular membranes express TNFα (Oh et al., 1999), and inhibition of TNFα signaling in an experimental laser-induced murine model of CNV reduced the size of CNV lesions and the extent of permeability (Shi et al., 2006). Furthermore, intravitreal injection of the drusen constituent Aβ induces prolonged transcriptional upregulation of TNFα in the neuroretina and transient upregulation in the RPE/choroid, suggesting that secretion of TNFα may occur in retinas of eyes with drusen (Liu et al., 2013).

A recent paper demonstrated that CEP-protein adducts can prime myeloid-derived cells to express pro-IL-1β (Doyle et al., 2012). CEP-protein adducts are products of oxidation found in greater abundance in the outer retinal tissues and sera of AMD patients than normal human donors. Induction of inflammasome assembly in bone marrow-derived macrophages via treatment with ATP resulted in production of IL-1β when the cells were first primed with HSA adducted to CEP, but not HSA alone. It was also shown that CEP-induced priming is mediated by TLR2, as macrophages from TLR2-null mice did not produce IL-1β when primed with CEP-adducted HSA and activated by ATP. In mice, RPE cells have been found to express low levels

of TLR2, which is upregulated by exposure to LPS (Fujimoto et al., 2010). Thus, it is possible that CEP-protein adducts may prime RPE cells.

Unlike pro-IL-1β, I detected both ASC and procaspase-1 under basal conditions. However, similar to pro-IL-1β, the levels of procaspase-1 were upregulated by priming, whereas ASC expression was largely unresponsive to priming. It remains unclear whether the expression of procaspase-1 is regulated in vivo as it is in vitro, but it is possible that priming is required for procaspase-1 expression in vivo as part of a mechanism to control inflammasome activity in the RPE. Based on studies in myeloid cells, it has been hypothesized that two signals are required for NLRP3 inflammasome activation, i.e. a priming signal to induce NLRP3 and pro-IL-1β expression and an activation signal to trigger inflammasome assembly. This level of control presumably exists to ensure that production of IL-1β, a very potent pro-inflammatory cytokine, only occurs in response to appropriate stimuli. Since RPE cells are not “professional” immune cells, it is possible that they may maintain even stricter control over the expression of inflammasome components than myeloid cells, requiring priming not only for expression of pro-IL-1β and NLRP3, but also upregulation of procaspase-1. This would not only prevent unintended activation of the NLRP3 inflammasome, but all of the inflammasomes.

Interestingly, both RPE cells and myeloid-derived cells such as macrophages are capable of phagocytosis. Whereas myeloid cells ingest foreign substances or pathogens, RPE cells primarily phagocytose the outer segment discs shed by photoreceptors. This RPE function is necessary for the replenishment of the chromophore 11-cis retinal, which is converted to all-trans retinal upon absorption of a photon (Sparrow et al., 2010). Photoreceptors do not express the enzymes necessary to convert all-trans retinal back to 11-cis retinal, whereas RPE cells do. Thus, all-trans retinal is transferred to an outer segment disc where it undergoes reduction to form all-trans retinol. Upon phagocytosis of the discs by the RPE, the enzymes LRAT, RPE65, and 11-cis retinol dehydrogenase convert all-trans retinol to 11-cis retinal, which is subsequently returned to the photoreceptor (Sparrow et al., 2010). Disc shedding and phagocytosis are

influenced by daily light-dark cycles, with shedding and phagocytosis peaking shortly after the onset of light and reaching their lowest levels during darkness (Grace et al., 1999). Since phagocytosis is a primary function of both RPE cells and myeloid-derived immune cells, it may be that the ability to express NLRP3 is a characteristic of phagocytes.

Activation of the NLRP3 inflammasome in RPE cells by lysosomal destabilization

As the expression of NLRP3 is not sufficient to activate the NLRP3 inflammasome, identification of the stimuli that activate the inflammasome in RPE cells is essential to evaluating the role that the NLRP3 inflammasome may play in AMD. Lysosomal destabilization has been shown to activate the NLRP3 inflammasome in myeloid cells, and I have demonstrated that destabilization of RPE lysosomes triggers inflammasome activation. To disrupt RPE lysosomes, I utilized the compound Leu-Leu-OMe, which accumulates in lysosomes and is converted to a membranolytic derivative by the intralysosomal enzyme DPP-I. Inhibition of DPP-I has allowed me to demonstrate that inflammasome activation induced by Leu-Leu-OMe is due to lysosomal disruption, rather than off-target effects of the molecule.

Studies using myeloid cells have indicated that lysosomal destabilization activates NLRP3 via a mechanism involving lysosomal proteases, specifically cathepsins B and L (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008; Rajamaki et al., 2010). Similarly, I have found that cathepsin B, and possibly cathepsin L, released by lysosomal membrane damage, are responsible for activating the NLRP3 inflammasome. A recent report demonstrated that NLRP3 inflammasome activation in glial cells by Aβ involves the degradation of NLRP10 by cathepsins B and L (Murphy et al., 2013). NLRP10 is a NLR family member that possesses a pyrin domain and a nucleotide-binding domain, but lacks leucine-rich repeats seen in other NLRs. NLRP10 negatively regulates inflammasome assembly by binding ASC and preventing its recruitment by NLRP3. During NLRP3 inflammasome assembly, NLRP3 recruits ASC

molecules and induces their oligomerization into a speck-like structure. NLRP10 antagonizes this process by binding ASC and preventing its association with NLRP3. Phagocytosis of Aβ by glial cells results in lysosomal destabilization and release of cathepsins B and L into the cytosol, where they degrade NLRP10, enabling binding of NLRP3 to ASC and assembly of the inflammasome. It is highly likely that lysosomal destabilization activates the NLRP3 inflammasome through a similar mechanism in RPE cells, either involving degradation of NLRP10 or an analogous inhibitory protein.

Drusen and lipofuscin as potential destabilizers of RPE lysosomes

The progression of AMD is thought to involve several sources of RPE lysosomal damage, including drusen, BLinD, and lipofuscin. Phagocytosis of isolated drusen deposits or drusen components such as the complement component C1Q has been reported to activate the NLRP3 inflammasome in myeloid cells (Doyle et al., 2012). In light of the fact that it is the insoluble physical nature of insoluble or particulate matter that is responsible for their ability to disrupt lysosomes and activate NLRP3, rather than their biochemical activity, it is likely that internalization of drusen, BLinD, or their constituents by RPE cells would also induce the NLRP3 inflammasome.

A recent study using rats found that intravitreal injection of the drusen constituent Aβ 1-40 led to the upregulation of genes including IL-1β, IL-18, and NLRP3 in the RPE/choroid (Liu et al., 2013). The specificity of this effect was demonstrated by the induction of IL-1β protein in the retinas and RPE of rats injected with Aβ 1-40, but not the non-aggregating reverse control peptide, Aβ 40-1. Levels of both IL-1β and IL-18 were significantly elevated in the vitreous of Aβ 1-40-injected rats, compared to their Aβ 40-1-injected counterparts. Consistent with the fact that that the cytokines were generated by the RPE, microglial activation was minimal. Despite the elevation in cytokine levels, no changes in neuroretinal thickness were noted in animals injected

with Aβ 1-40 up to 49 days post-injection, and there was no evidence of neovascularization or BrM disruption. The upregulation of NLRP3 and IL-1β observed in the RPE/choroid suggests that RPE cells are primed by exposure to Aβ 1-40. Additionally, the detection of IL-1β in the neuroretina and RPE, as well as the elevation of IL-1β and IL-18 in the vitreous, suggests that Aβ 1-40, and likely other drusen components, can activate the inflammasome in the RPE in vivo. One caveat with this study, however, is that it was not demonstrated that the cytokines had been processed into their mature forms. Furthermore, although microglial activation was minimal, it remains possible that a low level of microglial inflammasome activity could generate the cytokine levels observed in the study, rather than or in addition to the RPE.

It is important to note that the phagocytosis of outer segment discs takes place on the apical surface of the RPE, whereas drusen deposits are localized basolaterally. It seems unlikely that any phagocytosis occurs on the basolateral surface, as molecules that mediate photoreceptor outer segment phagocytosis, such as $\alpha_v\beta_5$ integrin and Mer tyrosine kinase, have an apical localization (Nandrot et al., 2012). It is speculated that drusen deposition is the result, at least in part, of exocytosis or transcytosis that occurs when the capacity for lysosomal degradation has been exceeded or impaired (Ambati et al., 2013; Krohne et al., 2010). Consistent with this notion, exosome marker proteins such as CD63 and LAMP2 have been shown to be present in drusen from AMD patients, but not in age-matched control individuals (Wang et al., 2009). In rats, reduction of lysosomal degradative ability using chloroquine results in the deposition of undegraded phagocytosed photoreceptor outer segments in the space between the RPE and BrM (Peters et al., 2006). In a like fashion, modification of proteins with the lipid peroxidation products 4-hydroxynonenal and malondialdehyde reduces their degradation in RPE lysosomes (Krohne et al., 2010). Modification of photoreceptor outer segment proteins with these products, followed by phagocytosis of the outer segments by ARPE-19 cells polarized on Transwell membranes, results in the release of undegraded outer segment proteins in the basolateral medium. It has been similarly observed in frog RPE that

residual products of lysosomal degradation are basolaterally exocytosed and accumulate between the RPE and BrM (Rungger-Brandle et al., 1987). Furthermore, presentation of latex particles to the apical surfaces of RPE cells leads to their ingestion and subsequent release on the basolateral side (Matsumura et al., 1985). These findings suggest that materials found in drusen may have originated from RPE lysosomes, with the corollary that drusen deposition results from impaired RPE lysosomal function. The presence in RPE lysosomes of molecules known to be constituents of drusen and/or the accumulation of undegraded material in dysfunctional RPE lysosomes may result in lysosomal membrane disruption and subsequent NLRP3 inflammasome activation. Additionally, although drusen accumulate on the basolateral surface, reticular pseudodrusen, which are also correlated with AMD progression, occur on the apical surface of the RPE and may be phagocytosed.

Lipofuscin, a pigmented, granular substance of heterogeneous composition consisting of the indigestible byproducts of lysosomal degradation, is also a characteristic of AMD. Lipofuscin accumulates in a number of cell types, especially postmitotic cells with substantial metabolic activity, including cardiac myocytes and some types of neurons (Sparrow and Boulton, 2005). A number of the compounds of RPE lipofuscin are derived from molecules present in phagocytosed outer segments. One such compound, the bisretinoid fluorophore A2E, is a major component of RPE lipofuscin (Schutt et al., 2007). However, the notion that A2E is a component of lipofuscin has recently been challenged, based on the finding that it does not colocalize with lipofuscin fluorescence in the human RPE (Ablonczy et al., 2013; Smith et al., 2013). If true, RPE lipofuscin nonetheless contains a number of bisretinoid molecules with similar chemical attributes as A2E, and therefore may have comparable effects on cells (Sparrow et al., 2013). A2E has been demonstrated to exhibit detergent-like properties and can destabilize lysosomal membranes (Schutt et al., 2002; Sparrow et al., 1999). Accordingly, a recent study reported that endocytosis of A2E by ARPE-19 cells in vitro activates the inflammasome, as evidenced by the formation of ASC speck-like complexes and the release of IL-1β from IL-1α-primed ARPE-19

cells (Anderson et al., 2013). Furthermore, the release of IL-1β from primed RPE cells is dependent on caspase-1 activity and is significantly inhibited by blockade of cathepsin B, suggesting that A2E-induced inflammasome activation occurs through cathepsin-mediated induction of the NLRP3 inflammasome. Thus, lysosomal destabilization represents a mechanism through which features associated with AMD may promote AMD pathology.

Effector functions of the NLRP3 inflammasome in AMD

Inflammasomes are capable of a number of effector functions, including the processing and release of mature IL-1β and IL-18, as well as caspase-1-mediated programmed cell death, or pyroptosis. My work demonstrates that activation of the inflammasome in primed RPE cells results in IL-1β maturation and release, as well as pyroptotic cell death. Pyroptosis mediated by caspase-1 may lead directly to RPE cell death, contributing to GA, whereas IL-1β may contribute to AMD via inflammation.

It should be noted that the levels of IL-1β released from RPE cells are modest compared to those released from myeloid-derived cells (Halle et al., 2008; Hornung et al., 2008). However, the inflammation associated with AMD is low-level and chronic in nature, and the relatively low levels of IL-1β generated by the RPE are consistent with this fact. The term para-inflammation has been used to describe a low-grade inflammatory response that is intermediate between the basal state and classic frank inflammation (Medzhitov, 2008). It is hypothesized that both classic inflammation and para-inflammation are mechanisms by which the body attempts to restore tissue homeostasis. Classic inflammation is a reaction to severe departures from tissue homeostasis, such as infection or overt tissue damage, whereas para-inflammation is a response to more moderate tissue dysfunction or stress that could result from exposure to noxious conditions, such as oxidative stress. Persistence of tissue stress or dysfunction may lead to a maladaptive state of chronic para-inflammation. Para-inflammation is believed to

contribute to pathological conditions such as atherosclerosis, obesity, type 2 diabetes mellitus, and neurodegenerative diseases (Xu et al., 2009). It is suspected that factors such as the age-related accumulation of oxidative damage in the outer retina, the deposition of drusen, the decline in RPE lysosomal degradative function over time, and other age-related retinal changes generate outer retinal tissue stress, resulting in chronic para-inflammation that contributes to AMD. My findings suggest that NLRP3 inflammasome activity in the RPE may be involved in para-inflammation in the outer retina. Lysosomal damage in the RPE, which is a form of tissue stress or dysfunction, may trigger the production of low levels of IL-1β via the inflammasome, promoting para-inflammation, and ultimately, AMD.

Comparison of NLRP3 inflammasome function in RPE versus myeloid cells

A majority of the studies on inflammasomes have been conducted in myeloid cells so it is of interest to identify the commonalities and differences between inflammasome function in RPE cells and in myeloid cells. My work indicates that RPE cells, similar to myeloid cells, require priming to induce expression of both pro-IL-1β and NLRP3, indicating that the two cell types may share signaling mechanisms responsible for upregulating pro-IL-1β and NLRP3. In vitro, however, NLRP3 is constitutively expressed in RPE cells, likely due to the effects of culture conditions as we did not detect expression in non-diseased cells in vitro.

Interestingly, my data demonstrated that priming also upregulates the expression of procaspase-1 in RPE cells in vitro, but procaspase-1 levels in myeloid cells have not been reported to respond to priming. It is important to note that a major function of myeloid cells is involvement in innate immunity whereas RPE cells serve a myriad of other essential roles. Therefore, it is possible that the requirement of priming for procaspase-1 upregulation may prevent unwarranted inflammasome activity in RPE cells. Also, RPE cells cannot be replaced if lost due to inflammation or pyroptotic cell death, whereas myeloid-derived cells are continually

replenished. Thus, the control of procaspase-1 levels via priming may restrict inflammasome activity in RPE cells only to situations where it is necessary, whereas this level of regulation is not essential in myeloid cells. That said, induction of procaspase-1 by priming in RPE cells has only been demonstrated in vitro, and this finding needs to be validated in vivo.

Regarding the mechanism of NLRP3 inflammasome activation, my findings support a model in which lysosomal destabilization induces NLRP3 inflammasome assembly via cathepsins, a mechanism similar to what has been described in myeloid cells (Halle et al., 2008; Hornung et al., 2008). However, lysosomal destabilization is only one of several proposed mechanisms for NLRP3 inflammasome activation in myeloid cells; others include potassium efflux and ROS production, and my results do not preclude the possibility that the other mechanisms function in RPE cells as well. In support of this possibility, activation of the NLRP3 inflammasome in RPE cells by *Alu* RNA elements has recently been shown to be mediated by the purinergic receptor P2X7 (Kerur et al., 2013). P2X7 functions as a ligand-gated cation channel that is opened by ATP. In myeloid cells, binding of extracellular ATP to P2X7 results in potassium efflux and subsequent induction of NLRP3 inflammasome assembly. The involvement of P2X7 in *Alu* RNA-mediated NLRP3 activation suggests that potassium efflux may trigger the NLRP3 inflammasome in RPE cells as well.

It remains unclear, even in the well-studied context of myeloid cells, how signals as distinct as potassium efflux, ROS generation, and lysosomal destabilization induce assembly of the NLRP3 inflammasome. It seems likely that these diverse stimuli activate mechanisms that feed into a common pathway that is the proximal inducer of NLRP3. It is not clear that all of these pathways function in RPE cells. It has recently been reported that oxidative stress induced by hydrogen peroxide or *tert*-butyl hydroperoxide causes ARPE-19 cells to undergo necrotic cell death, without triggering apoptosis or pyroptosis (Hanus et al., 2013). In these studies caspase-1 was not activated by treatment with hydrogen peroxide or *tert*-butyl hydroperoxide, suggesting that the ROS pathway of NLRP3 activation does not function in RPE

cells as it does in myeloid cells. Further research is warranted to evaluate the similarities and differences between the inflammasome priming and activation pathways in RPE cells and those in myeloid cells. Figure 11 illustrates the findings presented in this dissertation with those described in the literature for RPE and myeloid cells.

Involvement of the NLRP3 inflammasome in animal models of AMD

Recently, several studies have evaluated the contribution of the NLRP3 inflammasome to AMD-like pathology in mice. Importantly, modeling AMD in mice is problematic as mice lack a macula and therefore do not develop true AMD. Furthermore, mice are short-lived with an average lifespan of two years, and therefore the development of age-related pathology requires experimental manipulation that is unlikely to accurately reflect the temporal mechanism of the disease. Nonetheless, mice are receptive to genetic manipulations such as gene knockout, making them useful, albeit imperfect, model organisms for the study of AMD.

One study utilized mice with RPE-specific deletion of DICER1, which was demonstrated to lead to the intracellular accumulation of noncoding RNA transcripts expressed by the *Alu* retrotransposon (Tarallo et al., 2012). This accumulation of *Alu* transcripts in RPE results in degeneration mediated by the NLRP3 inflammasome and IL-18, but not by IL-1β. Deficiency in *NLRP3*, *ASC*, *Casp1*, or *IL18* prevents *Alu*-induced RPE degeneration, whereas the absence of *IL1R1*, the receptor that mediates IL-1β signaling, does not. The authors hypothesize that activation of the NLRP3 inflammasome in RPE cells by *Alu* RNA generates mature IL-18, which induces the RPE degeneration observed in GA. A subsequent study from the same group found that *Alu* RNAs prime RPE cells via TLR-independent NF-κB induction and activate the NLRP3 inflammasome via the $P2X_7$ receptor, a ligand-gated cation channel that responds to extracellular ATP, another NLRP3 agonist (Kerur et al., 2013). Binding of ATP to $P2X_7$ triggers potassium efflux, which in turn activates NLRP3 through an incompletely characterized

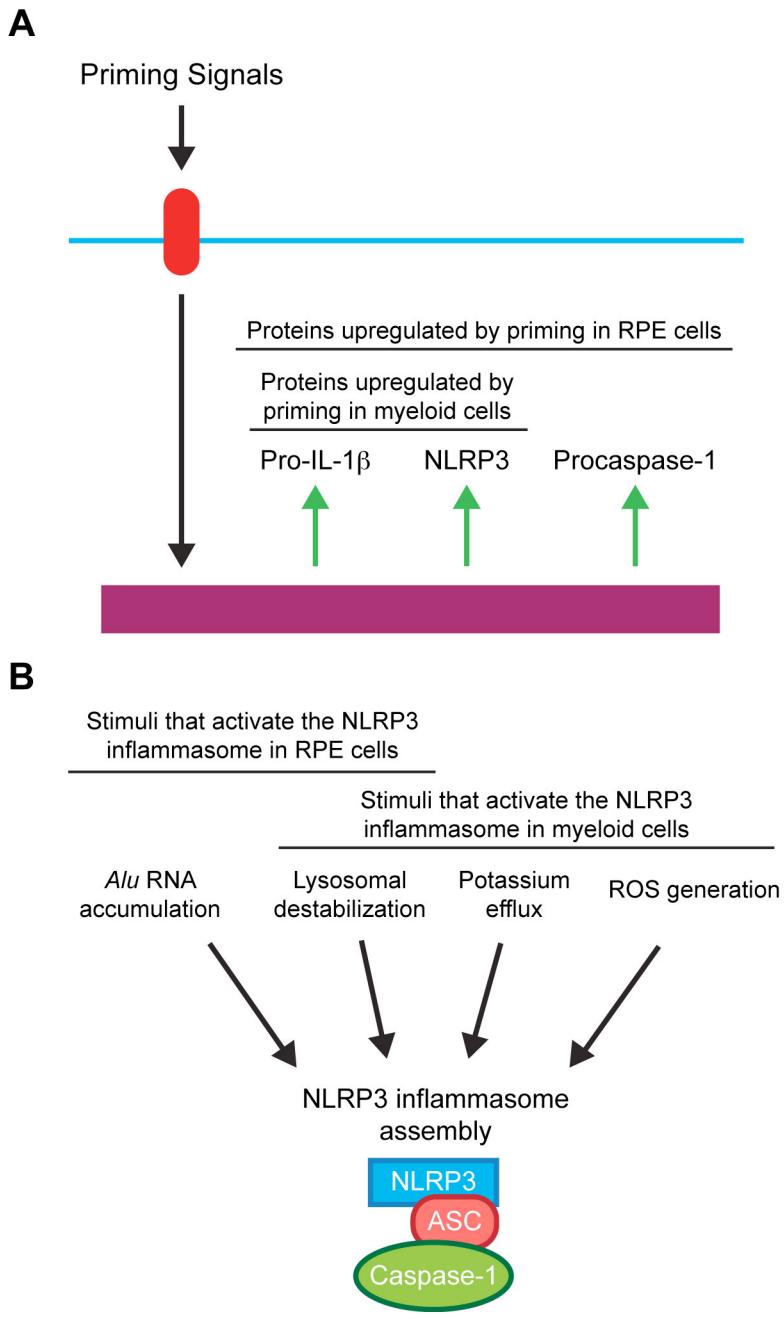

Figure 11. Schematic comparing NLRP3 inflammasome priming and activation in RPE and myeloid cells. (A) Priming signals induce upregulation of inflammasome-related proteins in myeloid cells and RPE cells. Previous research has determined that priming induces expression of pro-IL-1β and NLRP3 in myeloid cells. My findings indicate that priming RPE cells induces upregulation of procaspase-1, in addition to pro-IL-1β and NLRP3. (B) Activation of the NLRP3 inflammasome in myeloid cells has been demonstrated to occur in response to lysosomal destabilization, potassium efflux, and ROS production. My work shows that the NLRP3 inflammasome is activated by lysosomal destabilization in RPE cells. Other research has indicated that accumulation of *Alu* RNA transcripts in RPE cells also triggers NLRP3 inflammasome assembly, and it has not yet been determined whether this mode of NLRP3 activation functions in myeloid cells.

mechanism (Mariathasan et al., 2006; Schroder and Tschopp, 2010). It is likely that *Alu* transcripts activate NLRP3 through a similar mechanism, rather than through lysosomal destabilization, which occurs independently of $P2X_7$.

Whereas the latter study suggested that NLRP3 inflammasome activity in RPE cells might contribute to GA via IL-18, another group reported substantially different results. Using a model in which dry AMD-like pathology is induced by immunization of mice with CEP adducted to MSA, infiltrating macrophages in the retinas of mice were shown to stain for cleaved caspase-1, indicating inflammasome activation (Hollyfield et al., 2008). Furthermore, in a laser injury-induced mouse model of CNV, deletion of *NLRP3* or *IL18* exacerbated the neovascularization compared to wild-type mice, suggesting that these genes play a protective role against CNV (Doyle et al., 2012). On the other hand, deletion of *IL1R1* resulted in a trend toward reduction of CNV lesion volume compared to wild-type that did not reach statistical significance. Thus, the authors of this study hypothesize that macrophages are the primary source of NLRP3 inflammasome activity in AMD, and that NLRP3-mediated IL-18 release is protective against CNV. This suggestion is in direct contrast to the findings from the *Alu*-induced model, in which IL-18 generated via NLRP3 inflammasome activity contributed to RPE degeneration. These apparently conflicting results may due to the possibility that the NLRP3 inflammasome plays different roles in dry and wet AMD. However, it is important to note that the laser injury model of CNV is not fully representative of the neovascularization observed in wet AMD. Laser-induced CNV is more appropriately regarded as a model of acute wound healing that takes place in the presence of otherwise healthy RPE cells that likely are not primed to express NLRP3 (Marneros, 2013). Thus, given the previously reported role of the NLRP3 inflammasome in promoting epithelial repair via IL-18 (Dupaul-Chicoine et al., 2010; Zaki et al., 2010), it is reasonable to suspect that the function of the NLRP3 inflammasome in an acute wound-healing model does not reflect its role in AMD. That said, it could also be argued that *Alu* RNA transcript accumulation may not recapitulate the mechanism of dry AMD.

Another recent report utilizes a mouse model in which expression of vascular endothelial growth factor-A (VEGF-A) is heightened (VEGF-A^{hyper}) resulting in RPE abnormalities including loss of tight junctions (Marneros, 2013). Examination of the retinas of VEGF-A^{hyper} mice up to 24 months of age reveals progressive, age-associated RPE degeneration and concomitant photoreceptor degeneration. Sub-RPE deposits reminiscent of human dry AMD pathology were also noted, and these mice develop CNV at sites of RPE atrophy. Thus, this mouse model appears to mimic pathological features of both dry and wet AMD. Notably, the RPE of VEGF-A^{hyper} mice exhibit increased expression of NLRP3 and IL-1β, and the cleaved subunits of caspase-1 are detectable in the RPE/choroid, indicating inflammasome activation. Interestingly, deletion of *NLRP3*, *IL1R1*, or *IL18* did not inhibit the RPE degeneration observed in VEGF-A^{hyper} mice and did not prevent the development of CNV. However, CNV lesion numbers are reduced in VEGF-A^{hyper} mice deficient in NLRP3 or IL-1R, indicating that NLRP3 and IL-1β may contribute to CNV. Conversely, the absence of IL-18 results in a borderline significant increase in CNV lesions, suggesting a protective role for IL-18 in wet AMD. Once again, these conclusions must be interpreted with caution; although the VEGF-A^{hyper} model recapitulates a number of pathological features of AMD, there is no evidence that the underlying mechanism of this model accurately reflects the mechanisms of dry and wet AMD.

Future directions

AMD is a complex disease whose etiology remains elusive. Numerous factors are associated with AMD, such as the presence of drusen, BLinD, reticular pseudodrusen, and lipofuscin, but it is unclear if they are causative. Several lines of evidence, such as the presence of proinflammatory molecules in drusen and the link between CFH polymorphisms and AMD progression, imply a role for inflammation in the pathogenesis of AMD, but are nonetheless correlative. My findings suggest a mechanism that connects AMD-related RPE dysfunction,

involving lysosomal damage and exposure to proinflammatory molecules, with outer retinal inflammation and ultimately RPE degeneration via the NLRP3 inflammasome. Additional research is necessary to further evaluate this mechanism on a biochemical level in vitro, identify AMD-related molecules or deposits capable of priming or activating the NLRP3 inflammasome, and assess the role of the NLRP3 inflammasome in animal models of AMD-like pathology.

Biochemical characterization of the NLRP3 inflammasome pathway in RPE cells

Activation of the NLRP3 inflammasome is still incompletely understood in myeloid-derived cells, and even less is known about its induction in the RPE. The mechanisms that regulate the NLRP3 inflammasome in the RPE may differ from those in hematopoietic cells. Proteins involved in NLRP3 activation in myeloid cells may have analogous molecules in RPE cells, or the molecular pathways may be dramatically dissimilar. Therefore, it will be important to elucidate the biochemical mechanism of NLRP3 inflammasome activation in RPE cells. A convenient starting point would be to determine whether degradation of NLRP10 by cathepsins facilitates recruitment of ASC by NLRP3, as has been demonstrated in glial cells (Murphy et al., 2013). Additionally, potassium ion efflux and ROS generation have been found to induce the NLRP3 inflammasome in hematopoietic cells through mechanisms that appear to be independent of lysosomes. It would be of interest to determine whether NLRP3 can be induced in RPE cells by these stimuli.

Evaluation of NLRP3 inflammasome priming and activation by AMD-related insults

My findings have demonstrated that NF-κB agonists prime RPE cells, and that disrupting RPE lysosomes induces the NLRP3 inflammasome. However, it is not clear which molecular insults or changes associated with AMD, such as accumulation of drusen and other deposits, buildup of lipofuscin, AGE generation, and impairment of lysosomal degradative function, are capable of priming RPE cells or sufficiently damaging the integrity of the lysosomal membrane

to activate NLRP3. A2E has recently been demonstrated to induce IL-1β secretion from RPE cells via NLRP3 (Anderson et al., 2013), but the role of A2E as a lipofuscin constituent has been called into question (Ablonczy et al., 2013). CEP-protein adducts have been shown to prime myeloid cells (Doyle et al., 2012), but their effect on RPE cells has not been tested. Similarly, isolated drusen and individual drusen components such as Aβ and complement component C1Q have been found to activate the NLRP3 inflammasome in myeloid cells, but have not been evaluated in RPE cells (Doyle et al., 2012; Halle et al., 2008). It would be worthwhile to evaluate isolated drusen, individual drusen constituents, lipofuscin bisretinoids, CEP-protein adducts, and AGEs for their ability to prime or activate the NLRP3 inflammasome in RPE cells.

Assessment of the role of the NLRP3 inflammasome in murine models of AMD-like pathology

To demonstrate that the NLRP3 inflammasome mediates AMD pathogenesis in response to RPE lysosomal destabilization, it will be necessary to develop a mouse model of AMD-like retinal pathology induced by RPE lysosomal damage. This may be accomplished via subretinal injection of Leu-Leu-OMe or other lysosomotropic compounds, such as chloroquine or tamoxifen. To this end, I have conducted studies that demonstrate that tamoxifen induces RPE cell death via release of cathepsins B and L, suggesting that subretinal injection of tamoxifen may be a viable approach for modeling AMD-like pathology in mice. My work on tamoxifen-induced RPE cytotoxicity has been submitted for publication and is currently under review. The submitted manuscript is included in the Appendix of this dissertation. Once a suitable mouse model has been established, it will be important to evaluate the effects of this treatment on mice deficient in *Nlrp3*, *Asc*, or *Casp1* and compare their retinal pathology with that of wild-type mice.

Therapeutic implications

Whereas VEGF inhibitors are approved for neovascular AMD, there is currently no approved treatment for GA. However, the long-term safety profiles of VEGF inhibitors are just beginning to be revealed, and chronic inhibition of VEGF may have unanticipated side effects due to its role as a neuroprotectant for photoreceptors and as a survival factor for Muller cells, fenestrated vascular endothelium, and RPE (Ford et al., 2011; Saint-Geniez et al., 2009; Saint-Geniez et al., 2008). Thus, treatment options for AMD that target the NLRP3 inflammasome are likely to be of great benefit. Several biologic agents that target the IL-1 pathway are approved for use by the Food and Drug Administration. Anakinra, a recombinant IL-1 receptor antagonist, has been approved for treatment of rheumatoid arthritis, and the monoclonal anti-IL-1β antibody canakinumab and the "IL-1 trap" fusion protein rilonacept are approved for treatment of CAPS (Lachmann et al., 2011; Mitroulis et al., 2010). However, the potential role of the NLRP3 inflammasome in the pathogenesis of AMD, as well as the downstream cytokines involved, must be elucidated in order to determine whether targeting the IL-1 pathway will be effective.

Concluding remarks

As the American population continues to age, it is projected that nearly three million individuals will be affected by AMD by the year 2020 (Friedman et al., 2004). A more complete understanding of the mechanisms underlying AMD pathogenesis is essential if this growing public health challenge is to be addressed. The discovery that the NLRP3 inflammasome functions in RPE cells and can mediate IL-1β release from RPE cells is a key finding that may generate new therapeutic targets for AMD. However, research on inflammasomes in AMD remains nascent, and many questions remain regarding the role that the NLRP3 inflammasome plays in AMD. This uncertainty stems, at least in part, from the lack of accurate animal models of AMD. Nevertheless, the finding that the RPE lysosomal destabilization activates the NLRP3 inflammasome provides a mechanistic link between the classic features of AMD, drusen and

lipofuscin, and the inflammation and cytotoxicity that appear to contribute to RPE degeneration.

CHAPTER 5

MATERIALS AND METHODS

Immunohistochemistry of human retina

De-identified specimens from AMD and control human subjects were obtained from a tissue repository established in one of our laboratories at Schepens Eye Research Institute under IRB approval. Specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. For immunohistochemistry, sections were deparaffinized and antigen retrieval was performed with citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0, 95-100°C) for 10 minutes. Following two washes in phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO), slides were incubated with a mouse monoclonal anti-human NLRP3 antibody (1:100; clone Nalpy3-b; Enzo Life Sciences, Farmingdale, NY) or a mouse IgG1 isotype control antibody (1:100; Caltag, Carlsbad, CA) overnight at 4°C. The secondary antibody, a biotinylated horse anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA), was visualized via the avidin-biotin-alkaline phosphatase complex (ABC-AP) method (Vectastain ABC-AP Kit; Vector Laboratories) using the Vector Red Substrate (Vector Laboratories). Slides were counterstained with hematoxylin, dehydrated, and mounted with Permount medium (Fisher Scientific, Pittsburgh, PA).

RPE cell culture

Human ARPE-19 cells (American Type Culture Collection, Manassas, VA) were propagated as described previously (Ford et al., 2011). Cells were cultured in DMEM/F12 medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (Lonza), and 100 U/ml penicillin-100 µg/ml streptomycin (Lonza) (complete ARPE-19 medium) and passaged at a ratio of 1:2 to 1:4 using trypsin-EDTA (Life Technologies, Carlsbad, CA, or Lonza). For experiments, ARPE-19 cells were maintained in either 1% FBS or serum-free ARPE-19 medium, and transfections were

performed in antibiotic-free ARPE-19 medium.

Primary human fetal RPE (hfRPE) cells (Lonza) were propagated in Retinal Pigment Epithelial Cell Basal Medium (Lonza) supplemented with 5% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-100 µg/ml streptomycin (complete hfRPE medium). At confluence, hfRPE cells were passaged at a ratio of 1:2 to 1:4 using trypsin-EDTA (Life Technologies or Lonza). For experiments, hfRPE cells were seeded at passage 6-8 and were maintained in serum-free hfRPE medium.

Immunocytochemistry

ARPE-19 cells were cultured on Transwell membranes for four weeks in ARPE-19 medium with 1% FBS, as described elsewhere (Ford et al., 2011), to induce RPE polarization and tight junction formation. Monolayers were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and then incubated with a mouse monoclonal anti-human NLRP3 primary antibody (1:100; clone Nalpy3-b; Enzo Life Sciences) or a mouse IgG isotype control antibody (1:100; Life Technologies) overnight at 4°C. The secondary antibody, a biotinylated horse anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA), was visualized via the ABC-AP method (Vector Laboratories) using the Vector Red Substrate (Vector Laboratories). Nuclei were labeled with DAPI. Transwell membranes with attached monolayers were excised from their supports and mounted on glass slides for confocal microscopy (Leica Microsystems; Wetzlar, Germany).

Priming of ARPE-19 cells with NF-κB-inducing agents

ARPE-19 cells were seeded into 12-well plates (BD Biosciences, San Jose, CA) at a density of 2.6 x 10^5 cells/well in complete ARPE-19 medium. At confluence, the culture medium

was changed to serum-free medium and cells were treated with ultra-pure LPS from *Escherichia coli* 0111:B4 strain (InvivoGen, San Diego, CA), recombinant human IL-1α (R&D Systems, Minneapolis, MN), or recombinant human TNFα (PeproTech, Rocky Hill, NJ). Each agent was tested at 4 ng/ml or 50 ng/ml for 24 hours or 48 hours. Whole cell lysates were immunoblotted for pro-IL-1β.

NLRP3 knockdown

ARPE-19 cells were grown as described above and seeded into 6-well plates (BD Biosciences) at a density of 1.5 x 10^5 cells/well in antibiotic-free ARPE-19 medium. The following day, at approximately 40% confluence, cells were transfected with ON-TARGETplus SMARTpool siRNA against human NLRP3 or a non-targeting control SMARTpool (Dharmacon, Lafayette, CO). The target sequences of the siRNAs in each pool are provided in Table 1. The control siRNA pool was transfected at a total siRNA concentration of 100 nM using DharmaFECT 4 (Dharmacon), and the NLRP3 siRNA pool was used at 50 nM and 100 nM. At 24 hours post-transfection, cells were washed once with PBS, and complete ARPE-19 medium containing 4 ng/ml IL-1α was added to the wells to prime the cells. Cells were lysed at 72 hours post-transfection and immunoblotted for NLRP3.

NLRP3 overexpression and immunoblotting

To generate a positive control for NLRP3, lysates of HEK293T cells overexpressing the full-length transcript variant of NLRP3 fused to a DDK tag (identical to FLAG tag) and myc tag or transfected with a mock vector were purchased from OriGene (Rockville, MD). Immunoblotting for NLRP3 was performed on lysates of ARPE-19 and THP-1 cells, using the NLRP3- and mock-transfected HEK293T lysates as controls. Blotting for NLRP3 was followed by stripping

and re-probing first for α-tubulin and secondly for DDK.

Effect of priming on expression of pro-IL-1β and inflammasome components by ARPE-19 cells

ARPE-19 cells were seeded into 6-well plates at a density of 4.0 x 10^5 cells/well in complete ARPE-19 medium. At confluence, the cells were switched to serum-free ARPE-19 medium. The cells were primed with recombinant human IL-1α. For the dose curve, IL-1α was added to cells at concentrations of 1.56, 3.13, 6.25, 12.5, and 25 ng/ml IL-1α and incubated for 48 hours. For the time course, 4 ng/ml IL-1α was added to wells, and cells were lysed after 3, 6, 12, 24 or 48 hours. Cells were washed with ice-cold PBS, lysed, and immunoblotted for pro-IL-1β, NLRP3, ASC, and caspase-1.

Evaluation of hfRPE priming by IL-1α and LPS

hfRPE cells were seeded into 12-well plates at a density of 1.5 x 10^5 cells/well in complete hfRPE medium. Upon reaching confluence, the cells were switched to serum-free hfRPE medium containing IL-1α at concentrations of 3, 10, 30, or 100 ng/ml. Negative control cells received serum-free medium alone, and some cells were treated with 100 ng/ml ultra-pure LPS instead of IL-1α. After 24 hours, cells were washed with ice-cold PBS, lysed, and immunoblotted for pro-IL-1β and NLRP3.

Acridine orange staining of RPE lysosomes

ARPE-19 cells were seeded on sterile coverslips placed in a 6-well plate at a density of 3.0 x 10^5 cells/well in complete medium. The following day, cells were incubated with complete

medium containing 5 μM acridine orange for 30 minutes. Cells were washed twice with PBS, then treated with 1 mM Leu-Leu-OMe (Chem-Impex International, Wood Dale, IL, or Santa Cruz Biotechnology, Dallas, Texas) or control buffer for 30-45 minutes. Cells were then fixed with 4% paraformaldehyde for 30 minutes and washed three times with PBS. Coverslips were then mounted on glass slides using Vectashield Mounting Medium for Fluorescence (Vector Laboratories) and imaged on an Axioskop 2 mot plus fluorescent microscope (Carl Zeiss, Thornwood, NY).

Fluorescent detection of active caspase-1

ARPE-19 cells were seeded into 24-well plates (BD Biosciences) at a density of 5.0 x 10^4 cells/well in complete ARPE-19 medium, grown to confluence, and then changed to serum-free ARPE-19 medium with 4 ng/ml IL-1α. After 48 hours, the cells were pre-treated with the dipeptidyl peptidase I inhibitor Gly-Phe-CHN_2 (MP Biomedicals, Solon, OH) at a concentration of 10 μM, the cathepsin B and L inhibitor Z-FF-FMK (EMD Biosciences, San Diego, CA) at a concentration of 50 μM, or an equal volume of DMSO vehicle. After 30 minutes, the fluorescent labeled inhibitor of caspases (FLICA) probe specific for caspase-1 (FAM-YVAD-FMK; Immunochemistry Technologies, Bloomington, MN) was added to each well at the concentration recommended by the manufacturer, followed by the addition of 1 mM Leu-Leu-OMe. After a two-hour incubation at 37°C, 5% CO_2, cell nuclei were stained with Hoechst 33342 (Immunochemistry Technologies). After washing and fixing cells using wash buffer and fixative supplied by the manufacturer, cells were imaged via fluorescence microscopy.

For hfRPE cells, 48-well plates (BD Biosciences) were seeded at a density of 3.5 x 10^4 cells/well in complete hfRPE medium. At confluence, the cells were switched to serum-free hfRPE medium with or without 10 ng/ml IL-1α. After 24 hours, cells were treated with FAM-YVAD-FMK at the recommended dilution, followed by the addition of 1 mM Leu-Leu-OMe to

appropriate wells. Cells were incubated for two hours at 37°C, 5% CO_2, at which point cell nuclei were stained using Hoechst 33342. Cells were washed, fixed, and imaged by fluorescence microscopy.

For analysis of caspase-1 activation in polarized RPE monolayers, hfRPE cells were seeded onto 12-mm polyester Transwell membranes with 0.4 μm pores (Corning Incorporated, Tewksbury, MA) coated with 10 μg/ml laminin (Sigma-Aldrich). Cells were maintained in serum-free hfRPE medium, replenished twice weekly, for at least four weeks to allow them to form a polarized monolayer. The cells were then treated with serum-free medium with or without 10 ng/ml IL-1α. Following a 24-hour incubation, FAM-YVAD-FMK was added to all Transwells at the recommended dilution, and 1 mM Leu-Leu-OMe was added to appropriate wells. After a two-hour incubation at 37°C, 5% CO_2, cells were washed with buffer supplied by the manufacturer, then fixed with 4% paraformaldehyde for 30 minutes and washed three times with PBS. Transwell membranes were excised from their inserts and mounted on glass slides using ProLong Gold antifade reagent with DAPI (Life Technologies) and covered with a coverslip. Cells were then imaged via fluorescence microscopy.

ARPE-19 and hfRPE cells on plastic wells were imaged using a Nikon Eclipse TE2000-S microscope (Melville, NY). hfRPE cells on mounted Transwells were imaged with an Axioskop 2 mot plus fluorescent microscope. Quantification of green FLICA signal was performed using Adobe Photoshop. Blue nuclei in each image were counted manually, and the amount of green signal was normalized to the number of nuclei.

Immunoblotting for mature IL-1β in concentrated conditioned media

ARPE-19 cells grown to confluence in T75 flasks were primed with 15 ng/ml IL-1α for 48 hours, then treated with 1 mM Leu-Leu-OMe or control buffer for three hours. Conditioned media were harvested and concentrated using 15-ml Amicon centrifugal filter units (Millipore,

Billerica, MA) by spinning in an Avanti J-25I centrifuge (Beckman Coulter, Indianapolis, IN) using a JA25.50 rotor (Beckman Coulter) at 5,000 x g for one hour at room temperature. Following concentration, conditioned media were immunoblotted for IL-1β alongside a standard dilution of 0, 25, 50, 100, and 200 pg of recombinant human mature IL-1β (National Cancer Institute, Rockville, MD) as positive control. Goat anti-IL-1β (R&D Systems) was used as primary antibody at a 1:200 dilution, and HRP-linked rabbit anti-goat IgG (Santa Cruz) secondary antibody was used at a 1:5000 dilution.

Evaluation of inflammasome activation by lysosomal disruption

ARPE-19 cells were seeded onto 12-well plates at a density of 1.0 x 10^5 cells/well in complete ARPE-19 medium, grown to confluence and then changed to serum-free ARPE-19 medium with 4 ng/ml IL-1α. After 48 hr, Gly-Phe-CHN_2 (5 μM), Z-FF-FMK (50 μM), the caspase-1 inhibitor Z-YVAD-FMK (10 μM; BioVision, Mountain View, CA), or DMSO vehicle were added. After 30 minutes, 1 mM Leu-Leu-OMe was added to appropriate cells. Conditioned media were collected after three hours to assess cytokine secretion and lytic cell death.

For hfRPE cells, 12-well plates were seeded at a density of 1.5 x 10^5 cells/well in complete hfRPE medium. At confluence, the cells were changed to serum-free hfRPE medium with or without 10 ng/ml IL-1α. After 24 hours, cells were pre-treated with Gly-Phe-CHN_2 (10 μM), Z-FF-FMK (50 μM), the selective cathepsin B inhibitor CA-074-Me (50 μM; EMD Biosciences), or DMSO vehicle for 30 minutes. Then, 1 mM Leu-Leu-OMe was added to appropriate wells. After incubating at 37°C, 5% CO_2 for 3 hours, conditioned media were collected.

Quantification of cytokine secretion and cytotoxicity

ELISA was used to quantify IL-1β (BD Biosciences) and IL-18 (R&D Systems), and cytotoxicity was assessed by measuring the levels of lactate dehydrogenase (LDH) in conditioned media using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Each experimental condition was assayed in triplicate in three independent experiments. Percent LDH release was calculated as 100% × (experimental LDH − spontaneous LDH) / (maximum LDH − spontaneous LDH). Maximum LDH was represented by the LDH levels in wells completely lysed by two freeze-thaw cycles.

Immunoblot analysis of whole cell lysates

Cells were treated with lysis buffer (Cell Signaling Technology, Danvers, MA) containing a dissolved Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) and 2 mM PMSF. Protein concentrations were measured via the BCA assay (Thermo Scientific, Waltham, MA). Equal quantities of protein were separated via SDS-PAGE under reducing conditions and transferred to PVDF membranes (Millipore). Membranes were blocked overnight at 4°C. NLRP3 blots were blocked in Tris-buffered saline with 0.1% Tween (TBS-T) containing 5% (w/v) milk and 0.5% (w/v) bovine serum albumin (BSA); all other blots were blocked in 5% milk in TBS-T.

Membranes were then incubated in primary antibody diluted 1:1,000 in their respective blocking solution for two hours at room temperature. Primary antibodies used were mouse anti-NLRP3 (Nalpy3-b, Enzo Life Sciences), rabbit anti-caspase-1 (Cell Signaling), goat anti-IL-1β (R&D Systems), and rabbit anti-ASC (Enzo Life Sciences). The caspase-1 and IL-1β antibodies also recognize their uncleaved precursors. After three 10-minute washes in TBS-T, membranes were incubated for 1 hour at room temperature in secondary antibody diluted 1:10,000 in blocking buffer. HRP-linked secondary antibodies included sheep anti-mouse IgG (GE

Healthcare), donkey anti-rabbit IgG (GE Healthcare), and donkey anti-goat IgG (Santa Cruz). Following four more washes in TBS-T, proteins were visualized by enhanced chemiluminescence using SuperSignal substrates (Thermo Scientific). Membranes were stripped by incubation in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), and 0.1 M β-mercaptoethanol for 30 minutes at 55-60°C; re-blocked overnight at 4°C in 5% BSA in TBS-T; and re-probed with rabbit anti-GAPDH (Santa Cruz) to evaluate loading. NLRP3 blots were re-probed with mouse anti-α-tubulin (EMD Biosciences). To detect DDK-tagged NLRP3, blots were re-probed using mouse anti-DDK tag (OriGene).

Statistical analysis

Data are presented as the mean ± SEM of three independent experiments, unless otherwise indicated. To evaluate statistical significance, one-way analysis of variance was performed, followed by the Tukey-Kramer multiple comparisons test using the Prism 4 software package (GraphPad). A P-value of <0.05 was considered statistically significant.

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APPENDIX

NLRP3 Inflammasome and RIP Kinase Mediate Tamoxifen Toxicity of the Retinal Pigment Epithelium

Leo A. Kim[1,2], Dhanesh Amarnani[1,2], Wen Allen Tseng[1,2], Demetrios G. Vavvas[1], and Patricia A. D'Amore[1,2, 3*]

[1]Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, MA

[2]Schepens Eye Research Institute / Massachusetts Eye and Ear Infirmary, Boston, MA

[3]Department of Pathology, Harvard Medical School, Boston, MA

[*]: To whom correspondence should be addressed

Corresponding Author:

Name: Patricia A. D'Amore
Mailing Address: 20 Staniford St.
Boston, MA 02114
Telephone Number: 617-912-2559
Fax Number: 617-912-0128
Email address: patricia_damore@meei.harvard.edu

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ABSTRACT

Purpose. To evaluate the mechanism of tamoxifen-induced cell death in human cultured retinal pigment epithelial cells (RPE), and to investigate the relative contribution of cell death mechanisms including apoptosis, necroptosis, and pyroptosis.

Methods. Human RPE cells (ARPE-19 cells) were cultured until confluence and treated with tamoxifen; subsequent cell death was measured by detecting lactate dehydrogenase (LDH) release. Lysosomal membrane permeabilization was assessed using acridine orange staining. The roles of lysosomal enzymes cathepsin B and L were examined by blocking their activity. Caspase activity was evaluated by caspase-1, -3, -8, and -9 specific inhibition. Cells were primed with IL-1α and treated with tamoxifen and IL-1β production was quantified via ELISA. Caspase activity was verified with the fluorochrome-labeled inhibitor of caspases (FLICA) probe specific for each caspase. Necroptosis was evaluated using necrostatin-1 (Nec-1) to inhibit RIP1 kinase.

Results. Cell death occurred within two hours of tamoxifen treatment of confluent ARPE-19 cells, and was accompanied by lysosomal membrane permeabilization. Toxicity was shown to occur through both caspase-dependent and non-caspase-dependent cell death pathways. Blockage of cathepsin activity resulted in a significant decrease in cell death, indicating that lysosomal destabilization and cathepsin release are upstream of these cell death pathways. Treatment of ARPE-19 cells with caspase inhibitors and Nec-1 resulted in a near complete rescue from cell death.

Conclusions. Tamoxifen-induced cell death occurs through concurrent cell death mechanisms. Simultaneous inhibition of caspase-dependent and caspase-independent cell death pathways is

required to protect cells from tamoxifen. Inhibition of upstream activators such as the cathepsins may be a feasible approach to block multiple cell death pathways.

INTRODUCTION

Tamoxifen, a non-steroidal estrogen receptor antagonist, has been widely used in low dosages for the duration of five years as an adjuvant therapy for some forms of breast cancer. Although the current standard of care is five years of tamoxifen therapy, the global Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial recently showed that 10 years of tamoxifen reduced the risk of breast cancer recurrence, reduced breast cancer mortality, and reduced overall mortality. [1] Thus, it is likely that the standard of care will change, resulting in a longer period of tamoxifen therapy with a likely increase in tamoxifen-induced ocular toxicity.

Tamoxifen can lead to corneal toxicity, progression of cataracts, retinopathy, and neuropathy. The reported incidence of ocular toxic side effects among patients receiving tamoxifen ranges from 6.3% to 12%. The most visually significant aspect of tamoxifen toxicity is a maculopathy.[2] Tamoxifen is structurally similar to other drugs with well-known retinal effects including chloroquine, chlorpromazine, thioridazine, and tilorone. Although the RPE is thought to be the primary target of tamoxifen toxicity, recent reports have demonstrated that tamoxifen toxicity also affects photoreceptors as well. [3] Ultrastructural lesions associated with these agents may appear as crystalloid inclusions in the neuroretina or as crystalloid bodies within the RPE, which are thought to disrupt lysosomal function. RPE cells serve a critical role in the maintenance of photoreceptors, phagocytizing the outer segment tips of photoreceptors, which are then digested within lysosomes. RPE dysfunction is thought to play a role in a variety of retinal diseases including age-related macular degeneration (AMD), tamoxifen retinopathy, chloroquine retinopathy, central serous retinopathy, as well as a variety of inherited retinal disorders.

Inflammasomes are a class of multiprotein complexes that activate caspase-1 by facilitating the cleavage of procaspase-1 to active caspase-1, which induces the proteolytic maturation of the pro-inflammatory cytokines IL-1β and IL-18. The NLRP3 inflammasome can

be activated by a diverse array of signals. [4] Many of these signals activate NLRP3 by destabilizing lysosomes. Crystalline or insoluble materials such as cholesterol crystals and amyloid-β can activate NLRP3 in phagocytic myeloid-derived cells by disrupting phagolysosomes. [5-8] It has recently been demonstrated that the RPE express components of the NLRP3 inflammasome, which plays a role in animal models of AMD through lysosomal destabilization or accumulation of *Alu* RNA due to DICER1 deficiency in the RPE. [9, 10] We hypothesize that medications such as tamoxifen can disrupt lysosomal membranes, leading to the activation of the NLRP3 inflammasome, release of the pro-inflammatory cytokine IL-1β, and pyroptosis.[11]

Necroptosis is characterized by the activation of RIP-1 and RIP-3 kinase and is triggered by a variety of stimuli including TNF, DNA damage, and viral infection. [12-15] Cellular components or endogenous adjuvants such as high mobility group protein B1, uric acid, galectins, and thioredoxin released as a consequence of cellular demise promote an inflammatory response with activation of inflammasomes, cytokine production, inflammatory cell recruitment, and T-cell activation.[16] Necroptosis has been defined as caspase-independent cell death with a necrotic phenotype that can be prevented by the specific RIP1 inhibitor necrostatin-1 (Nec-1). [17, 18] Necroptosis has been demonstrated to occur in T lymphocytes, photoreceptors, astrocytes, and neurons and has been suggested to be involved in myocardial infarction.[19-23]

Here, we report on the involvement of multiple cell death mechanisms in tamoxifen-induced toxicity of the RPE. Specifically, we examined the roles of inflammasome-mediated cell death, the extrinsic and intrinsic pathway of apoptosis, and RIP kinase-mediated necroptosis.

MATERIALS AND METHODS

ARPE-19 Cell Culture

Human ARPE-19 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM/F12 medium (Lonza, Walkersville, MD) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (Lonza, Hopkington, MA), and 100 U/ml penicillin-100 µg/ml streptomycin (Lonza, Hopkington, MA) in a humidified incubator at 37°C, 10% CO_2 and passaged at a ratio of 1:2 to 1:4 using 0.25% Trypsin-EDTA (Invitrogen).

Phalloidin staining of ARPE-19 cells

ARPE-19 cells were seeded on coverslips placed in a sterile 12-well plate. Cells were serum-starved overnight then treated with 20 µM tamoxifen or control media without serum. The cells were fixed at 15, 30, 45, 60, 90, 120 and 180 minutes using 3% paraformaldehyde, permeabilized using 0.1% Triton X-100, and blocked for 60 minutes by washing with PBS between steps. The cells were then incubated with Alexa Fluor 594 phalloidin (1:100) in blocking buffer for 20 minutes, washed, and the coverslips mounted using mounting media with DAPI (Prolong Gold Antifade Reagent with DAPI, Life Technologies). Images were taken using a Leica AF6000 microscope.

Acridine orange staining of RPE lysosomes

ARPE-19 cells were seeded in a 48-well plate with complete growth media at a density of 3.3×10^4 cells/ well. Cells were grown to confluence and serum-starved overnight before the experiment. The following day, cells were incubated with serum-free medium containing 5 µM Acridine Orange (Immunochemistry Technologies, Bloomington MN) for 30 minutes at 37^0 C. Cells were washed twice with PBS followed by treatment with 20 µM tamoxifen or control buffer for 120 minutes. Images of wells were taken over the course of 2 hours to evaluate the progression of lysosomal destabilization with time.

Priming of ARPE-19 Cells with IL-1α and quantification of IL-1β secretion and cytotoxicity

ARPE-19 cells were seeded onto 12-well plates at a density of 1.0 x 10^5 cells/well in complete growth media. At confluence, cells were then changed to serum-free medium and pre-treated with 10 ng/ml IL-1α for 48 hours. Cells were then treated with 30 μM tamoxifen and control buffer in the presence or absence of 30 μM necrostatin and 10 μM Z-YVAD-FMK for 2 hours. IL-1β was quantified via ELISA (BD Biosciences) as described below. Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) in conditioned media, using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) as described below. Each experimental condition was assayed in triplicate in at least three independent experiments.

Quantification of IL-1β

ARPE-19 cells were seeded onto 12-well plates in complete media and grown to confluence. The cells were primed for 48 hours with 10 ng/mL IL-1α. After 48 hours, the cells were treated with tamoxifen (30 μM), DMSO vehicle or 1 mM L-leucyl-L-leucine methyl ester (Leu-Leu-OMe; Chem-Impex International, Wood Dale, IL). Conditioned media were collected after three hours and IL-1β was quantified via ELISA (BD Biosciences). Values were corrected for release of pro-IL-1β due to cytotoxicity using the method described by Miao and colleagues, in which levels of lactate dehydrogenase (LDH) in conditioned media were measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).[24]

Assessment of cytotoxicity

The condition media were collected from the treated wells and percent cell death was quantified measuring the lactate dehydrogenase in the condition media using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Condition media from the wells treated with control conditions were used as negative control. Percent LDH release was

calculated as 100% x (experimental LDH- spontaneous LDH)/ (maximal LDH- spontaneous LDH). The LDH levels in wells completely lysed by two freeze-thaw cycles represented maximum LDH.

Fluorescent detection of active caspase-1, 3, 8, and 9

ARPE-19 cells were seeded on to coverslips in complete growth medium and grown to confluence. The media were changed to serum-free and the cells incubated overnight. The cells were pretreated with FLICA for two hours (protected from light then incubated with tamoxifen (20 μM) or an equal volume of DMSO for controls at 37°C, 10% CO_2 for 60, 90, and 120 minutes). The fluorochrome-labeled inhibitor of caspases (FLICA) probes specific for caspase-1, 3, 8, and 9 (FAM-YVAD-FMK, FAM-DEVD-FMK, FAM-LETD-FMK, and FAM-LEHD-FMK; Immunohistochemistry Technologies, Bloomington, MN) were added to each well at the concentration recommended by the manufacturer. Following incubation for 60, 90, and 120 minutes, cells were washed and the cell nuclei were stained with Hoechst 33342 (Immunohistochemistry Technologies). Cells were then washed and fixed using wash buffer and fixative supplied in the FLICA kit, and imaged using a Leica AF6000 microscope.

Western blotting of RIP kinases in ARPE-19 cells

ARPE-19 cells were lysed using 1X RIPA buffer (Cell Signaling, Boston, MA) containing Complete Mini EDTA-free Protease Inhibitor Tablet (Roche, Indianapolis, IN), 2 mM phenyl methanesulfonyl fluoride (PMSF), and 2 mM sodium orthovanadate (NaOV) and then sonicated on ice. Protein concentrations were measured using BCA assay (ThermoScientific, Waltham, MA). Equal concentrations of protein were separated using 10% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked for one hour at room temperature in 3% bovine serum albumin (BSA). The membrane was then incubated with anti-RIP3 primary antibody (Abcam) (1:1000 in 1% BSA) overnight at

4°C. After three 10-minute washes in Tris-buffered saline with 0.1% Tween 20 (TBS-T), membranes were incubated for one hour at room temperature in secondary antibody (1:10,000 in blocking buffer). HRP-linked secondary antibodies included anti-mouse IgG (GE Healthcare, Pittsburgh, PA) and anti-rabbit IgG (GE Healthcare). Following three more washes in TBS-T, proteins were visualized by enhanced chemiluminescence using SuperSignal substrates (Thermo Scientific). Membranes were stripped by incubation in Stripping buffer (1 M Tris-HCl, pH 6.8; 10% (w/v) SDS; 0.7% beta-mercaptoethanol) for 25 minutes at 55°C to 60°C, re-blocked at room temperature in 3% BSA in TBS-T and re-probed with rabbit anti-RIP1.

Statistical Analysis

Data are presented as mean ± SEM of at least three independent experiments. Statistical significance was evaluated using a one-way analysis of variance followed by post-hoc Tukey-Kramer multiple comparison tests, using the Prism 6.0 software package (Graphpad). Adjusted P-values < 0.05 were considered statistically significant.

RESULTS

Tamoxifen induces cell death and lysosomal destabilization

Treatment of ARPE-19 cells with tamoxifen for two hours at various concentrations (10-30 μM) induced cell death in a dose-dependent fashion, as detected by release of LDH (Figure 1A). Two-hour exposure of cells to 10 and 15 μM tamoxifen had little effect on cell viability whereas exposure of the ARPE-19 cells for 20, 25 and 30 μM tamoxifen induced 40-70% LDH release. Treated cells exhibited features of cell death including swollen cell bodies, shrinkage, blebbing, and detachment from the culture substrate (Figure 1B). Visualization of the cytoskeleton by phalloidin staining displayed a loss of actin stress fibers by 60 minutes (Figure 1C). The active metabolite of tamoxifen, 4-OH-tamoxifen, similarly induced LDH release and cell death in ARPE-19 cells in a dose dependent manner (data not shown). All subsequent studies in this report utilized 20 μM tamoxifen unless stated otherwise.

Lysosomal integrity was assessed by acridine orange staining, which labels nucleic acids green and lysosomes red. Untreated ARPE-19 cells displayed punctate red-orange structures, characteristic of intact lysosomes (Figure 2). Treatment with tamoxifen resulted in loss of lysosomal staining after 20 minutes, indicative of lysosomal destabilization. Lysosomal destabilization occurred prior to LDH release (cell death), suggesting that lysosomal destabilization is upstream of tamoxifen-mediated cytotoxicity in these cells.

Lysosomal cathepsins in tamoxifen cytotoxicity

Lysosomal membrane permeabilization results in the release of cathepsins, which have been implicated in the activation of the NLRP3 inflammasome as well as in the initiation of apoptosis and necroptosis. Blockade of the lysosomal cathepsins B and L with the cathepsin B/L inhibitor Z-FF-FMK [25] led to a significant decrease in cell death as measured by LDH release (6 ± 2% compared to 73 ± 10% with tamoxifen treatment alone) (Figure 3A). There was no statistically significant difference between cathepsin B/L inhibition and concurrent inhibition of

caspases and RIP1 kinase. The decrease in cell death resulting from the inhibition of the cathepsins was greater than any other inhibitor alone, suggesting that cathepsin release from lysosomes occurs upstream and prior to the initiation of cell death pathways.

To elucidate the role of cathepsin B versus cathepsin L, specific cathepsin B and L inhibitors, using the cathepsin B inhibitor CA-074-Me and cathepsin L inhibitor Z-FY(t-Bu)-DMK were used (Figure 3C). Whereas blocking either cathepsin (B or L) protected the cells from tamoxifen toxicity, treatment with the cathepsin L inhibitor led to a significantly greater inhibition of cell death than the cathepsin B inhibitor (25 ± 6% versus 40 ± 0.4% LDH release). These data suggest a greater role for cathepsin L than cathepsin B in mediating tamoxifen cytotoxicity, suggesting that these cell death pathways depend upon the activity of cathepsin L. As expected, concurrent inhibition with the individual cathepsin B and L inhibitors resulted in near complete inhibition of cell death (Figure 3C).

Caspases and RIP1 kinase in tamoxifen cytotoxicity

We have previously shown that ARPE-19 cells express components of the NLRP3 inflammasome and activate caspase-1 upon lysosomal destabilization. [9] In addition, caspase-1 is the effector caspase in a form of programmed cell death referred to as pyroptosis.[26] The role of caspase-1 in tamoxifen toxicity of ARPE-19 cells was investigated using the caspase-1-specific inhibitor Z-YVAD-FMK. Two hours of treatment with 20 μM tamoxifen resulted in 73 ± 10% LDH release; the application of the caspase-1 inhibitor led to a significant decrease in cell death (51 ± 5%) (Figure 3A). Pan-caspase inhibition with Z-VAD-FMK did not yield additional protection from cell toxicity over that seen with caspases-1 inhibition alone.

Nec-1, a potent and selective inhibitor of programmed cell necrosis, targets the activity of receptor-interacting protein 1 (RIP1) kinase [17]. Concurrent treatment with pan-caspase, caspase-1, caspase-3 inhibitors and Nec-1 resulted in near complete inhibition of cell death after two hours of treatment with tamoxifen (Figure 3A, B). Activation of caspase 1 and 3 by

tamoxifen was further verified by FLICA assay (Figure 4, rows 1 and 2). These results implicate the activation of multiple caspase and non-caspase dependent cell death pathways with application of tamoxifen.

In order to investigate the possible involvement of the RIP kinase pathway in ARPE-19 cells, the expression of RIP1 and RIP3 kinase, the primary mediators of necroptosis were examined in ARPE-19 cells. Western blot analysis revealed the expression of both RIP1 and RIP3 in ARPE-19 cells (supplemental Figure 1).

Apoptosis occurs primarily through a caspase-9 dependent mechanism

Apoptosis, a form of cell death that eliminates damaged cells in a controlled manner, is characterized by caspase activation, chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies that are phagocytized by surrounding cells in order to prevent inflammation. Two main pathways that are involved in apoptosis, the extrinsic death receptor mediated pathway and the intrinsic or mitochondrial pathway, both converge on common executioner caspases (caspases-3, -6, and -7); we therefore investigated the relative role of the intrinsic and extrinsic pathways of apoptosis. The extrinsic pathway depends upon activation of the initiator caspase-8, which was probed with the caspase-8 specific inhibitor Z-LETD-FMK. The intrinsic or mitochondrial pathway of apoptosis was examined using the caspase-9 specific inhibitor Z-LEHD-FMK.

Inhibition of caspase-8 alone did not significantly decrease cell death in cells treated with tamoxifen, whereas caspase-9 inhibition alone led to a statistically significant decrease in cell death, indicating that apoptosis primarily occurs through the intrinsic pathway and is dependent upon mitochondrial dysfunction. Co-treatment with Nec-1 and caspase-8 inhibition yielded a near complete inhibition of cell death, implying that caspase-8 is activated by tamoxifen and that its inhibition results in cell death due to the activation of the RIP kinase pathway. Activation of both caspase 8 and 9 was verified with FLICA assay (Figure 4, row 3 and 4).

Caspase-1 activation leads to production of IL-1β

Lysosomal destabilization has been previously shown to induce the formation of the NLRP3 inflammasome and activation of caspase-1 with subsequent release of mature IL-1β and pyroptosis. In order to evaluate the activation of the NLRP3 inflammasome and caspase-1 activation, we examined mature IL-1β production in ARPE-19 cells treated with tamoxifen. ARPE-19 cells were primed with IL-1α for 24 hours and the level of mature IL-1β in the conditioned media was quantified by ELISA as previously described.[9]

Tamoxifen-treated ARPE-19 cells produced 17.55 pg/ml of IL-1β. Inclusion of Nec-1 did not reduce IL-1ß levels (15.26 pg/ml) (Figure 5). In contrast, treatment with the caspase-1 inhibitor Z-YVAD-FMK led to a significant decrease in the production of IL-1β, which was not further decreased by the addition of Nec-1 (6.38 and 7.75 pg/ml respectively) (Figure 5). These results are consistent with NLRP3 inflammasome activation by tamoxifen. Unprimed ARPE-19 cells exposed to tamoxifen did not produce mature IL-1β, but did exhibit cell death (data not shown), indicating that cell death associated with caspase-1 activity is independent of its ability to produce mature IL-1β.

DISCUSSION

Tamoxifen toxicity of the retina is believed to be mediated by damage to the RPE through disruption of lysosomes.[3] We examined the mechanisms of tamoxifen toxicity in ARPE-19 cells and demonstrated that tamoxifen-induced lysosomal destabilization is associated with the activation of a number of cell death pathways. We observed lysosomal destabilization in ARPE-19 cells within 20 minutes of tamoxifen treatment, a time prior to cell death. We found that cathepsins released upon lysosomal destabilization play a role in initiating multiple cell death mechanisms: necroptosis, apoptosis, and pyroptosis.

Lysosomal membrane permeabilization has been demonstrated to release hydrolases such as the cathepsins that have been demonstrated to participate in apoptosis. Upon their release into the cytosol, cathepsins cleave the pro-apoptotic Bcl-2 family member Bid, resulting in its activation, and degrade anti-apoptotic protein Bcl-2 proteins, triggering the intrinsic pathway of apoptosis. [27] [28] Similarly, inhibition of lysosomal cathepsins has been demonstrated to block NLRP3 signaling and caspase-1 activation. [5] [29] [30] In particular, cathepsin B inhibition has been implicated in caspase-independent cell death, and cathepsins B and L have been found to mediate inflammasome activity in myeloid-derived cells and ARPE-19 cells. Cathepsins have also been shown to play a role in necroptosis, specifically, in caspase-compromised conditions; necroptosis can be arrested with application of cathepsin B inhibitor CA-074-OMe.[31] Similarly, we have shown that combined cathepsin B and L inhibition leads to complete inhibition of cell death, and that the cathepsin release occurs upstream of multiple cell death mechanisms.

Caspases mediate both the extrinsic and intrinsic pathways of apoptosis. The extrinsic apoptosis pathway is activated when death receptor ligands such as TNF-α bind their receptor, which then recruits FADD. This interaction forms a death-inducing complex, which recruits the initiator procaspase-8, leading to its cleavage and activation. Activated caspase-8 then activates caspase-3 and other effector caspases that mediate the cell death pathway. The

intrinsic pathway is induced at the level of the mitochondria by cellular stresses (such as DNA damage, oxidative stress, or endoplasmic reticulum stress). The initiator procaspase-9 forms a complex with the mitochondrial protein cytochrome and apoptotic protease-activating factor (APAF-1) to form the apoptosome. [32] Active caspase-9 cleaves and activates effector caspase-3.

We demonstrated a significant role for the executioner caspase-3 in tamoxifen cytotoxicity. Further, we have shown a primary role for the caspase-9 dependent intrinsic pathway, an observation that is indicative of mitochondrial dysfunction. This is in agreement with the finding that necroptosis induces mitochondrial dysfunction, ROS formation, and release of pro-apoptotic proteins of the Bcl-2 family. [33] Although the extrinsic pathway, which is caspase-8 dependent, does not seem to play a primary role in tamoxifen-mediated toxicity, others have reported that inhibition of caspase-8 activates RIP1 kinase.[34] Consistent with this finding, co-treatment with Nec-1 and a caspase-8 inhibitor led to near complete inhibition of cell death, greater than Nec-1 alone.

Necroptosis depends on the activity of the serine/threonine kinase RIP1, an adaptor kinase that functions downstream of death domain receptors and forms a complex with RIP3 to activate necroptosis. Exposure of RPE cells to tamoxifen led to the activation of RIP1 and the initiation of necroptosis. As previously demonstrated in photoreceptors in a model of retinal detachment, co-treatment with caspase inhibitors and Nec-1 effectively suppresses tamoxifen mediated cell death of the RPE.[12, 15, 20, 35, 36]

Pathological activation of the NLRP3 inflammasome is characteristic of a variety of disease conditions in which lysosomal destabilization results from phagocytosis of crystals or insoluble aggregates.[6, 7] For instance, silicosis and asbestosis are mediated by NLRP3 inflammasome activation in myeloid cells induced by silica crystals and asbestos fibers. Similarly, monosodium urate crystals cause gout via NLRP3 activation, and cholesterol crystals trigger NLRP3 during the development of atherosclerosis. [4] The NLRP3 inflammasome has

been detected in RPE of eyes from patients with geographic atrophy and age-related macular degeneration [9], and has been postulated to play a role in their pathogenesis. Similarly, tamoxifen retinopathy is associated with the presence of retinal crystalline deposits and displays features similar to geographic atrophy with loss of the RPE and subsequent associated retinal atrophy.

The role of the NLRP3 inflammasome in tamoxifen toxicity of ARPE-19 cells is evidenced by the rapid activation of caspase-1 with subsequent production of mature IL-1β and pyroptosis following tamoxifen treatment. Caspase-1 contributes to cell death through induction of pyroptosis; however, inhibition of caspase-1 significantly but only partially, reduced cell death, indicating that while caspase-1 did contribute to tamoxifen-mediated RPE cell death, cell death was not mediated predominantly by pyroptosis. Thus, multiple cell death mechanisms contribute to tamoxifen toxicity of the RPE.

For therapeutic intervention, however, simultaneous inhibition of multiple cell death pathways may not be practical. Instead, targeting a common factor upstream of all pathways involved would likely yield better outcomes with regard to safety and efficacy. As we have found that cathepsins B and L are key mediators of tamoxifen-induced RPE cytotoxicity, these proteases may be viable candidates for the development of pharmacological inhibitors. While inhibition of only one of these enzymes results in partial reduction of tamoxifen toxicity, blockade of both cathepsins completely protects RPE cells from tamoxifen-induced cell death *in vitro*, and this was achieved using a single peptide-derived inhibitor. This overlap in substrate specificity may allow for tamoxifen retinopathy to be treated or prevented by a single drug targeting both cathepsins B and L.

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FIGURES

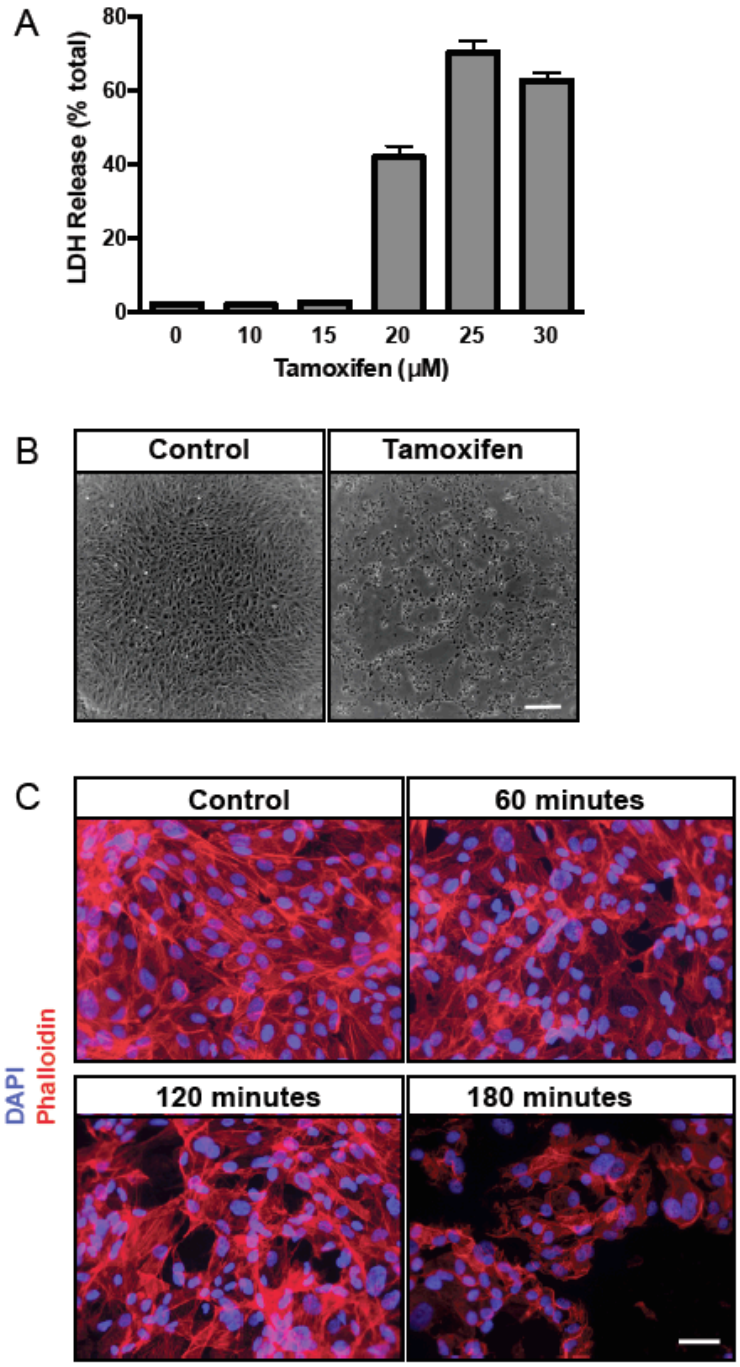


Figure 1. Tamoxifen cytotoxicity of ARPE-19 cells. (A) Increasing doses of tamoxifen led to ARPE-19 cell death and LDH release. (B) Bright field microscopy of control and tamoxifen- (20 μM) treated ARPE-19 cells after 2 hours of exposure. Cells appeared round and shrunken, and were detaching from the tissue culture plate (scale bar = 200 μm). (C) Phalloidin staining of actin fibers (red) and DAPI staining of cell nuclei (blue) reveals progressive loss of stress fibers, through time in ARPE-19 cells treated with tamoxifen (scale bar = 25 μm).

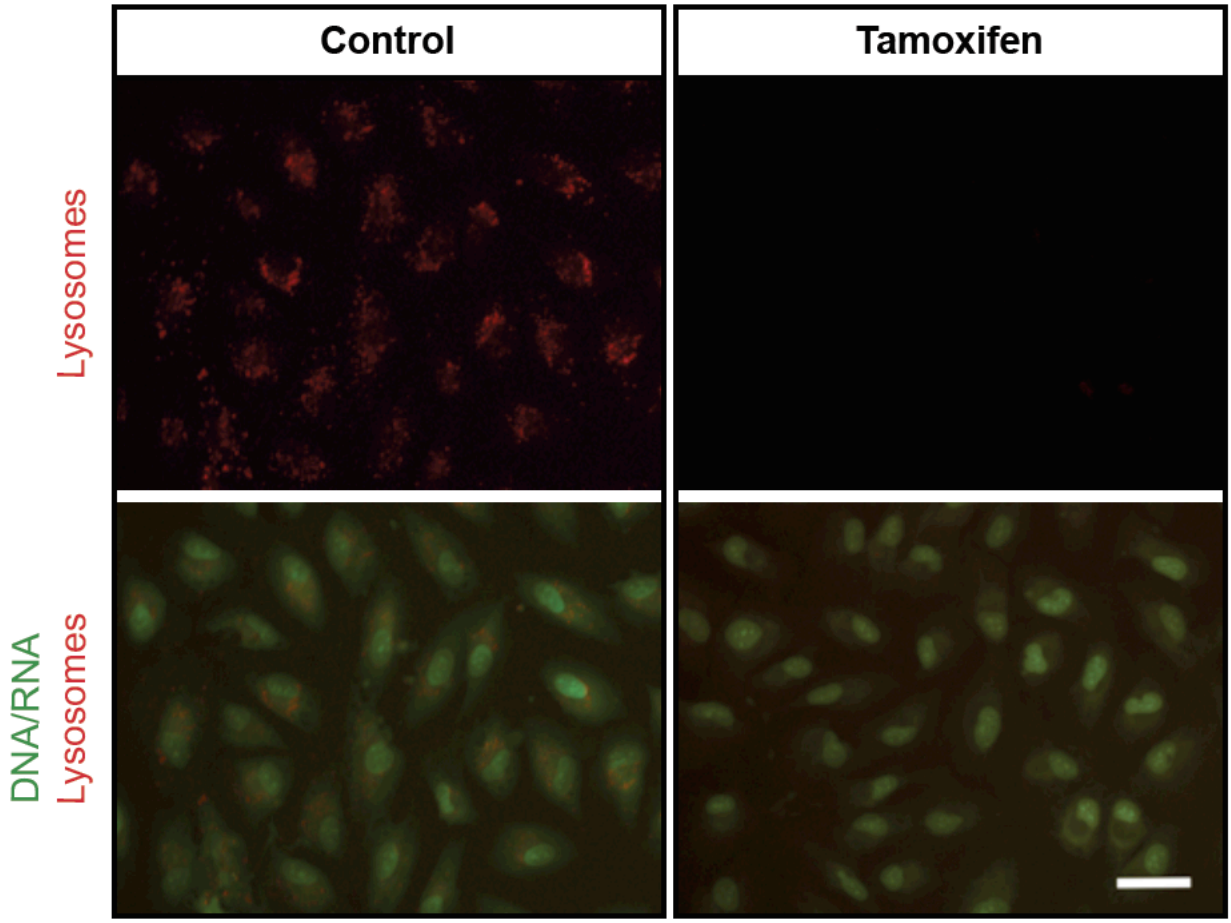


Figure 2. Tamoxifen-induced lysosomal destabilization in ARPE-19 cells. ARPE-19 cells were stained acridine orange prior to exposure to 20 μM tamoxifen. Fluorescence microscopy revealed lysosomes (red) and DNA or RNA (green). Lysosomal destabilization was evident within 20 minutes of tamoxifen exposure (scale bar = 10 μm).

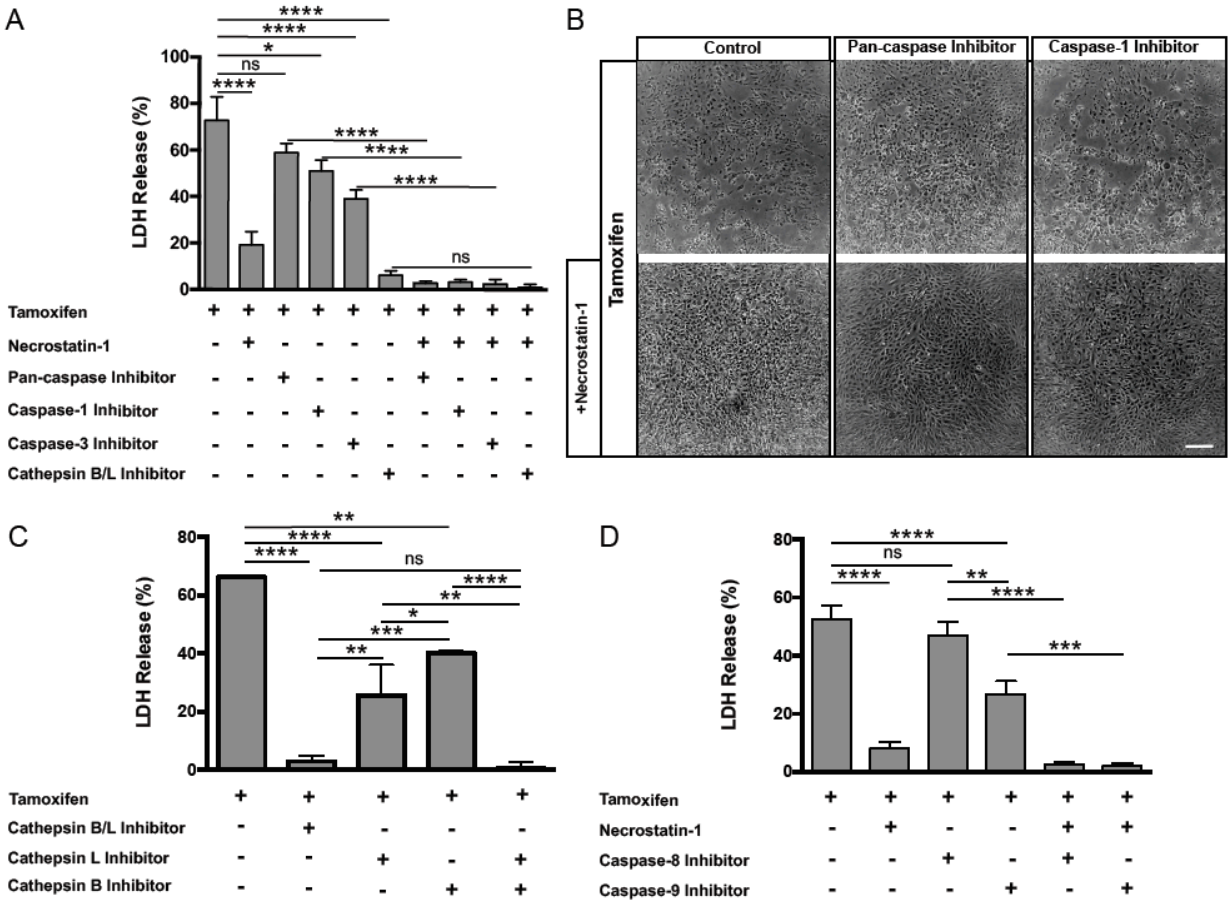


Figure 3. Pathways of tamoxifen-mediated ARPE-19 cell death. (A) ARPE-19 cells were treated with 20 μM tamoxifen for 2 hours, then treated with Nec-1 (20 μM) plus Z-VAD-FMK, a pan-caspase inhibitor (10 μM); Z-YVAD-FMK, a caspase-1 inhibitor (10 μM); Z-DEVD-FMK, a caspase-3 inhibitor (10 μM); Z-FF-FMK, a cathepsin B/L inhibitor (20 μM) alone or in the indicated combinations. (B) Brightfield images of ARPE-19 cells treated as in (A). Upper row represents cells treated with individual inhibitors without Nec-1. Cells were rounded, shrunken and were detaching from the substrate. Treatment with Nec-1 alone inhibited cell death, but the cells still appear distressed (bottom row, first panel). Simultaneous treatment with a pan-caspase inhibitor and Nec-1 or a caspase-1 inhibitor and necrostatin-1 resulted in near complete inhibition of cell death (bottom row, second and third panel). (C) ARPE-19 cells were treated with 20 μM tamoxifen for 2 hours, then treated with cathepsin B/L (Z-FF-FMK, 20 μM), specific cathepsin B (CA-074-Me) and cathepsin L (Z-FY(t-Bu)-DMK) inhibitors (20 uM) alone or in combination. (D) ARPE-19 cells were treated with 20 μM tamoxifen for 2 hours, then treated with Nec-1 (20 μM) plus Z-LETD-FMK, a caspase-8 inhibitor (10 μM); Z-LEHD-FMK, a caspase-9 inhibitor alone or in combination. Adjusted P-value ** < 0.05, *** < 0.005, **** < 0.0001

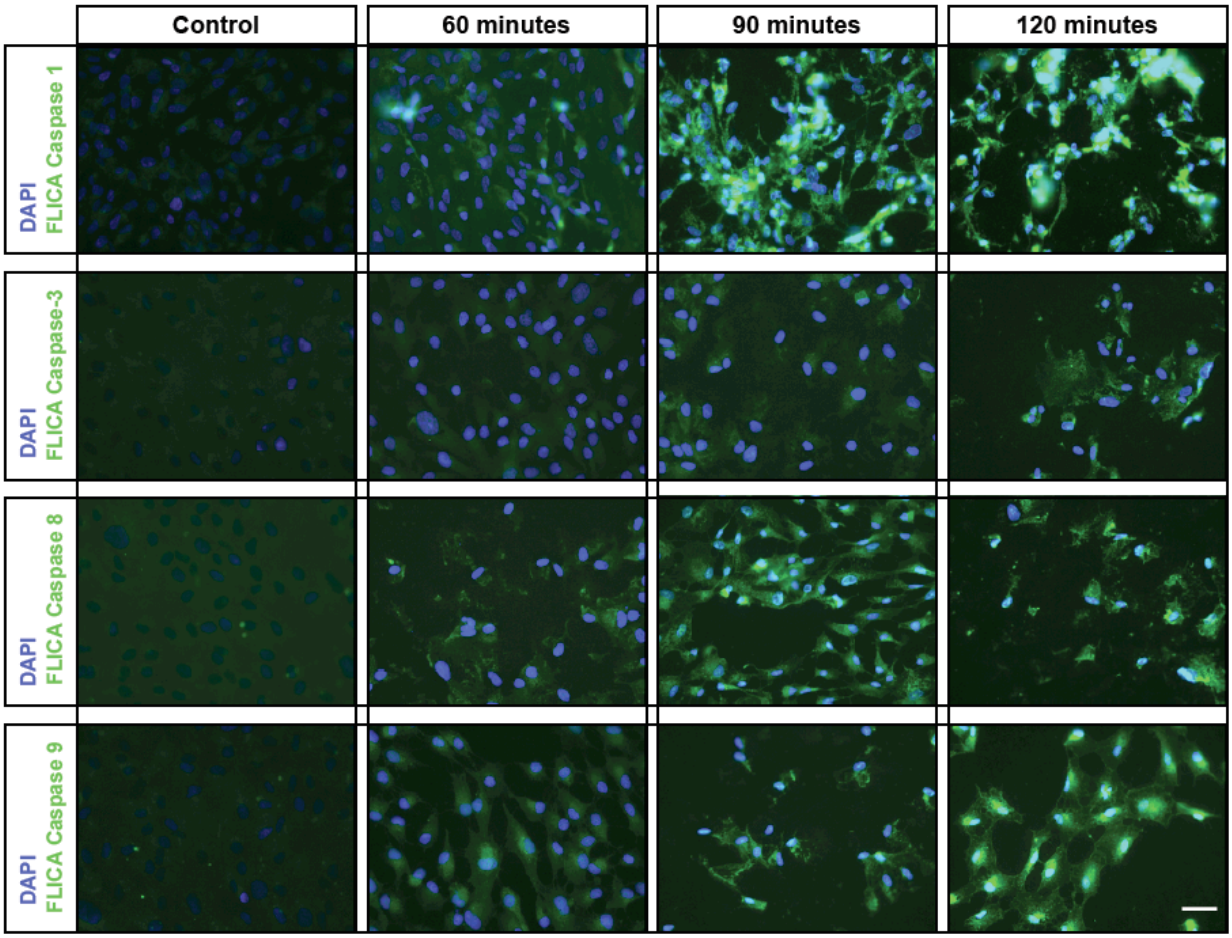


Figure 4. Caspase activation in tamoxifen-treated ARPE-19 cells. Fluorochrome-labeled inhibitors of caspases (FLICA) were used to examine the activity of caspases-1, -3, -8, and -9 in ARPE-19 cells treated with tamoxifen. (Row 1) Caspase-1 activity was noted 60 minutes after exposure to tamoxifen and was increased at 90 and 120 minutes. (Row 2) Elevated levels of caspase-3 activity were measured 90 minutes after application of tamoxifen. (Row 3,4) Caspase-8 and caspase-9 activity was observed 60 minutes after application of tamoxifen with increasing levels of fluorescence at 90 and 120 minutes.(scale bar = 25 μm).

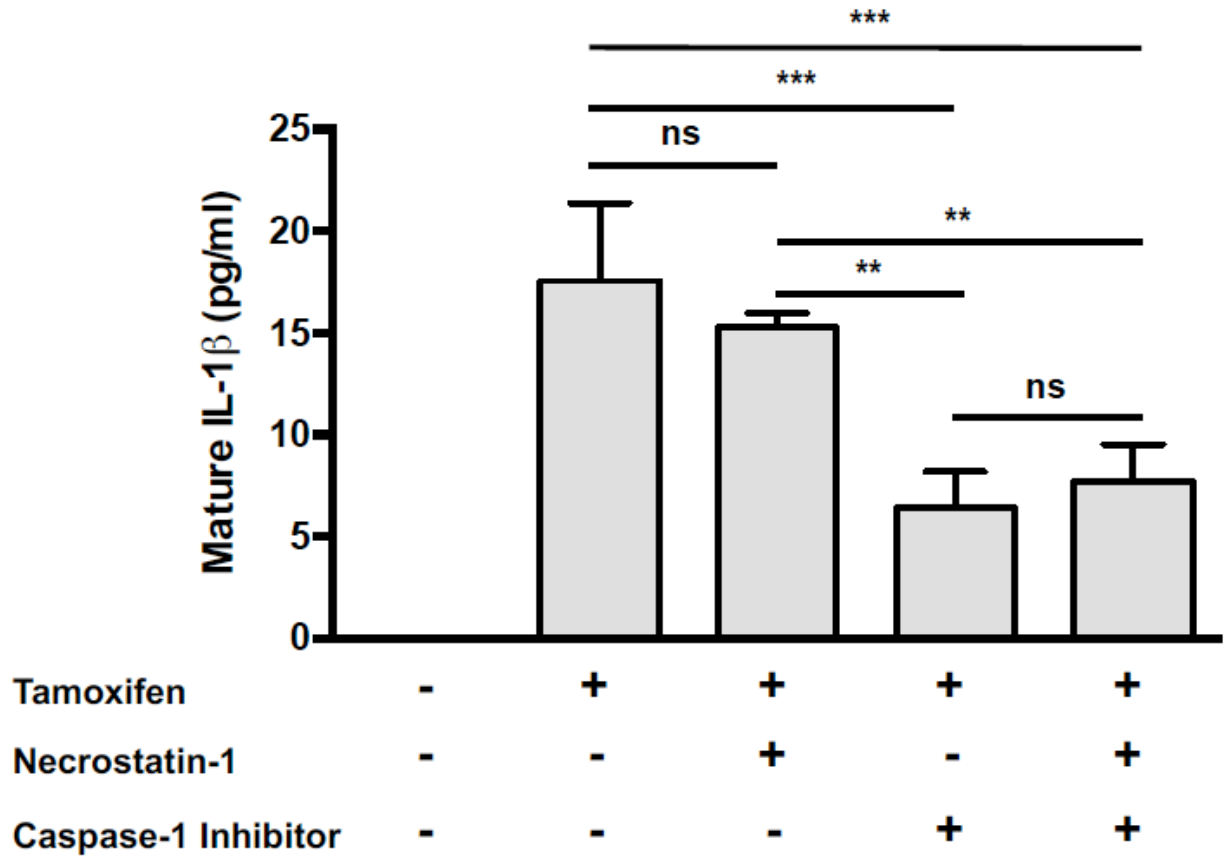
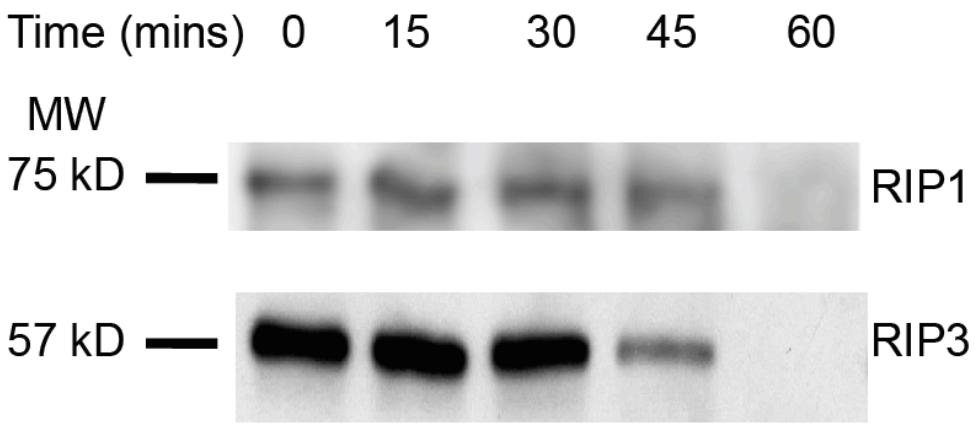


Figure 5. IL-1β production in primed ARPE-19 cells treated with tamoxifen. Primed ARPE-19 cells produced mature IL-1β when treated with tamoxifen, which was not affected by the addition of Nec-1. Treatment with a caspase-1 specific inhibitor significantly decreased production of IL-1β, which was not affected by the addition of Nec-1. Adjusted P-value ** < 0.05, *** < 0.005.



Supplemental Figure 1. RIP1 and RIP3 expression in ARPE-19 cells. RIP1 and RIP3 expression in ARPE-19 cells treated with tamoxifen 0, 10, 15, 30, 45, and 60 minutes after application of tamoxifen. Protein expression of RIP1 and RIP3 decreases after 45 minutes of exposure to tamoxifen.